Impact of emerging diseases on amphibian assemblages

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Abstract. Recent decades have seen unprecedented loss of global biodiversity with amphibians among the most affected species. Emerging infectious diseases have being linked to declines and may result in very rapid extinctions, with chytridiomicoses and ranaviroses cited as diseases with the greatest impact. However, demonstration of a link between population declines and infectious pathogens is not always straightforward and becomes harder if pre-outbreak data are not available. With this thesis I combine observational, experimental and modelling approaches to analyse the spread and impacts of emerging agents on amphibian assemblages. In Iberia, the first case of *Bd*-infection and consequent mass mortality was documented in 1997 in Sierra de Guadarrama (Spain), leading to the decline of common midwife toads (*Alytes obstetricans*). In Portugal, impacts were first recorded in 2009 in Serra da Estrela and here described. *Bd* led to a collapse of midwife toads in high altitude areas. However, despite being regarded as a highly susceptible host to *Bd*, midwife toads seem to exhibit strong variation in the prevalence of infection across small geographic scales. Although present at lower elevations, an altitudinal envelope prevented chytrid from causing mortality among this amphibian assemblage. Through ongoing monitoring, I detected the asynchronous emergence of a second pathogen: a *Ranavirus*. The new virus had the capacity to infect multiple hosts, leading to massive annual die-offs in different taxa, life stages and across the altitudinal range. Data recorded prior to the first outbreak, as well as observations at a comparable site with similar geoclimatic features, illustrated the collapse of the amphibian community in relation to potential drivers. Laboratory experiments and field observations showed that *S. salamandra* larvae were resistant to the disease chytridiomycosis despite sharing *Bd*-positive sites with dying *Alytes*. These results contrast with other higher elevation systems where salamander larvae often overwinter. This prolonged exposure increases the chances of infection and supports the idea that the impact of a pathogen can be mediated by host life history. The mediation of a pathogen impact by the host natural history and/ or particular behaviour was also demonstrated on newts. The lethal effects of *Ranavirus* annual exposure on the newt population were amplified by host phenology causing a strongly imbalanced adult sex ratio. Modelling showed how behaviour may influence recovery. Following mass mortality, population recovery will be faster if there is an even sex ratio. *Bd*-mediated declines and extinctions may occur in species with certain life history traits, whereas *Ranavirus* CMTV-like strains seem to have a broader impact across amphibian species. Good surveillance and monitoring are key steps for effective management and conservation of wild populations. Sustainable conservation of wild amphibian assemblages is dependent on long-term population persistence and co-evolution with these lethal pathogens.

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Chapter I

General Introduction

General Introduction

1.1. Biodiversity crisis and amphibian declines

Recent decades have seen unprecedented loss of global biodiversity with scientists from different fields warning about the most critical environmental problems (Pimm *et al*., 2014; Ceballos *et al*., 2015). The rate of extinction for species in the 20th century was up to 100 times higher than it would have been without anthropogenic impact, suggesting that Earth's biota is entering a sixth mass extinction (Pimm *et al*., 2014; Ceballos *et al*., 2015). Wildlife and their habitats cannot support the pressure that humanity is placing on the planet, with almost one-fifth of extant vertebrate species classified as Threatened (Hoffmann *et al*., 2010). Vertebrates play vital roles in ecosystems occupying most major habitat types (Terborgh, 1988), yet habitat destruction, alteration and fragmentation are highest on the list of threats (Ehrlich & Ehrlich, 1981; Tilman *et al*., 1994). Deforestation has increased dramatically in tropical regions (e.g., ~30% in the Brazilian Amazon between 2001-2004; INPE, 2004) with alarming effects on the emission of greenhouse gases (Santilli *et al*., 2005). This has direct implications on climate change (Thompson, 1980; Bonan, 2008) which in turn, leads to an increased extinction risk with over 50% of the species disappearing - in more dramatic scenarios - by 2050 (Thomas *et al*., 2004). The introduction of alien and invasive species has been pointed as the next most important cause of extinction after habitat loss, when non-native species prey on or compete with the natives (Vitousek *et al*., 1996, 1997).

Amphibians are particularly remarkable survivors, having endured four waves of mass extinction, the last one being at the end of the Cretaceous and leading to the extinction of non-avian dinosaurs (Sheehan & Fastovsky, 1992; Wake & Vredenburg, 2008). The three extant orders (Anura, Caudata and Gymnophiona) comprise more than 7000 described species (Frost, 2013) now facing a new wave of extinction (Barnosky *et al*., 2011) with 41% of the species at risk of disappearing (Hoffmann *et al*., 2010).

But it was only in 1989, at the First World Congress of Herpetology, that the scientific community became aware of a new global crisis (Stuart *et al*., 2004). What some thought of as strange, local phenomena where populations were disappearing or found in much lower numbers, was in fact being replicated in many other countries. First reports showing population declines dated as early as the 1970s, coming from the western United States (Kagarise Sherman & Morton, 1993; Drost & Fellers, 1996), Puerto Rico (Burrowes *et*

al., 2004), and northeastern Australia (Czechura & Ingram, 1990). Amphibian fauna was also quickly disappearing in Central and South America in places such as Costa Rica (40% at one site in the 80's; Pounds *et al*., 1997) Ecuador and Venezuela, with montane species being particularly affected (Pounds & Crump, 1994; Pounds *et al*., 1997; Young *et al*., 2001; Ron *et al*., 2003). The need for a comprehensive picture of the extent of these amphibian declines prompted a global assessment coordinated by IUCN. The results from this Global Amphibian Assessment (GAA) demonstrated that amphibians were far more threatened than other vertebrates, such as birds or mammals (Stuart *et al*., 2004).

Losses of amphibian populations and species are occurring at unprecedented rates driven largely once more by habitat loss. However, there are many cases where the habitat is protected and amphibians continue to disappear, a scenario that may result from climate change, pollution, or even synergism between multiple factors (Stuart *et al*., 2004; Wake & Vredenburg, 2008; Blaustein *et al*., 2011). A series of amphibian die-offs in Australia during the 1980s and 1990s in pristine areas suggested a pathogen as causative agent (Laurance *et al*., 1996). That pathogen was confirmed to be a chytrid fungus later described in 1999 (Berger *et al*., 1998; Longcore *et al*., 1999).

The discovery of this new pathogen generated a scientific debate over its emergence with two hypotheses being supported by observational evidence: 1) climate change, causing drying of the habitat leading to crowding of amphibians in remnant humid areas and increasing the transmission of the pathogen (Pounds *et al*., 1999); 2) a wave-like spread with exposure of naïve host populations to this pathogen suggesting introduction of disease into these areas from elsewhere (Daszak *et al*., 1999; Lips *et al*., 2008), which was supported by the discovery of chytrid fungus-infected frogs in the international trade (e.g., Pessier *et al*., 1999; Berger *et al*., 1998). In any case both hypotheses are almost entirely the product of anthropogenic environmental change (Daszak *et al*., 2001).

1.2. Infectious diseases as a threat to biodiversity

Although not ranked among the leading threats for amphibians, emerging infectious diseases (EID) have being linked to declines and may result in very rapid extinctions (Daszak *et al*., 2003), almost certainly underestimating the role of disease in species extinctions (Smith *et al*., 2006). EIDs are those that have increased in incidence, virulence or geographic range, have shifted hosts or have recently evolved new strains (Daszak *et al*., 2000), with chytridiomicoses and ranaviroses in the spotlight as the diseases with the greatest impact on amphibians (Duffus & Cunningham, 2010). The increasing number of wildlife EIDs may

reflect greater vigilance and documentation, but causal factors driving the emergence of wildlife diseases (e.g. international movement of people, livestock and other animals; Pimm *et al.*, 1995; McMichael *et al*., 1999) can also be implicated. Nevertheless, there seems to be a consensus: as long as human activities continue to alter the environment and ecosystems, it is likely that we will witness further outbreaks of novel and emerging pathogens in wildlife (Dobson & Foufopoulos, 2001).

Historically, wildlife diseases have been neglected and only considered when agriculture or human health was being compromised. Additionally, the paradigm at the time didn't consider parasites as a possible driver of host extinction. However, in recent years this trend has been changing with wildlife diseases now being taken more seriously (McCallum & Dobson, 1995; Hess, 1996). Pathogens are also becoming widely recognized as posing major conservation threats to animal health, and are responsible for episodes of mass mortality leading to declines of wildlife populations (Daszak *et al*., 2000; Smith *et al*., 2006; Fisher *et al*., 2012). For instance, animal-infecting pathogens, like the microsporid fungi were associated with the decline of bee populations (vanEngelsdorp *et al*., 2009), and *Pseudogymnoascus destructans*, a fungus that causes the fatal disease white-nose syndrome (WNS) has decimated bat populations in North America (Blehert *et al*., 2009). The majority of disease emergences may primarily result from a change in the ecology of host, pathogen, or both (Schrag & Wiener, 1995).

Chytrids and ranaviruses were shown to have been responsible for outbreaks and amphibian declines being recorded in several protected and non-protected areas (see below). But other diseases may also be responsible for massive mortality in amphibian assemblages, although the overall knowledge of the pathogens and their dynamics are still scarce. This is the case, for example, for *Amphibiocystidium* (causative agent of dermocistidiose; González-Hernández *et al.*, 2010), the trematode parasite, *Ribeiroia ondatrae*, which has been shown to cause deformities of some amphibian metamorphs (Johnson *et al*., 1999; Rohr *et al*., 2008), and some bacterial agents of the Chlamydiaceae family capable of infecting anurans (Martel *et al*., 2012b) and caudates (Martel *et al*., 2012a).

Demonstration of a link between population declines and infectious pathogens is not always straightforward (Daszak *et al*., 2003), requiring identification of the causative pathogen, isolation of the pathogen in culture, pathological evidence, and clear evidence that the die-offs are the cause of declines, which becomes hard if pre-outbreak data is not available.

1.2.1. Chytridiomycosis

Chytridiomycosis is an infectious disease caused by pathogenic fungi of the genus *Batrachochytrium* that is intimately linked to declines and extinctions of hundreds of species of amphibians (Skerratt *et al*., 2007). This is a widely studied amphibian disease and to date, two agents are known to lead to infection: *Batrachochytrium dendrobatidis* (*Bd*) (Longcore *et al*., 1999) and the more recently described *B. salamandrivorans* (*Bsal*) (Martel *et al*., 2013).

1.2.1.1. *Batrachochytrium dendrobatidis*

The amphibian chytrid fungus grows on skin and produces aquatic zoospores. After invading the epidermis, chytrid zoospores lead to proliferation of keratin cells (hyperplasia and hyperkeratosis) in post-metamorphic individuals (Berger *et al*., 1998; Kilpatrick *et al*., 2010). The mechanism by which *Bd* causes morbidity and mortality seems to be by disrupting skin function, leading to a consequent loss of homeostasis and – ultimately – heart failure (Voyles *et al*., 2009). In larvae, *Bd* infects the mouthparts (where keratin occurs during most of tadpole life), which may lead to depigmentation and sometimes causing damage to the mouth (Marantelli *et al*., 2004; Knapp & Morgan, 2006). Clinical signs of chytridiomycosis include anorexia, abnormal posture, lethargy and loss of righting reflex. Externally it is still possible to observe an abnormal epidermal desquamation, and in some particular cases, ulceration, bleeding into the skin, muscle or eye. However, these signs are not specific and the disease cannot be diagnosed clinically (Berger *et al*., 2000). The diagnosis of chytridiomycosis requires confirmation using other laboratory techniques, such as molecular detection, histopathology, or even by isolation and culture of the fungus.

Able to infect over 500 species of amphibians, the most serious manifestations of chytridiomycosis have been described for the Neotropical region of the Americas. The disease is thought to be involved in the disappearance of dozens of species of harlequin toads (genus *Atelopus*), particularly in higher elevation areas (La Marca *et al*., 2005), as well as a rapid loss of amphibian biodiversity in El Cope, Panama, affecting eight families of frogs and salamanders (Lips *et al*., 2006). *Bd* effects have also been felt in other different tropical environments, temperate and mountain, from the Caribbean, North and South America (especially the Andean region), Australia, and even in non-tropical regions, such as in Spain and Portugal, in Europe (Fisher *et al*., 2009; Catenazzi *et al*., 2011; Rosa *et al*., 2013).

Southern European countries host the highest European amphibian biodiversity, and the Iberian Peninsula in particular shows a high number of endemisms (about 30%; Pleguezuelos *et al*., 2002; Loureiro *et al*., 2008). There, the first case of *Bd* infection and

consequent mass mortality was recorded in 1997 in Sierra de Guadarrama in Spain, leading to the decline of common midwife toads (*Alytes obstetricans*) (Bosch *et al*., 2001). In Portugal the presence of *Bd* was reported in 2005 by Garner *et al*. (2005), but the first signs of impact were only recorded in 2009 in Serra da Estrela, where hundreds of recently metamorphosed common midwife toads were found dead at the edge of a pond, leading to a collapse of the population in high altitude areas (Rose *et al*., 2013; see Chapter II). However, whilst some species appear to have completely disappeared after an outbreak of chytridiomycosis, others persisted without (apparently) being affected, despite the prevalence of low levels of infection (Retallick *et al.*, 2004; Newel *et al*., 2013).

This chytrid fungus can be highly pathogenic and even lead to mortality after low levels of infection (Skerratt *et al*., 2007). But contrastingly, it has also been repeatedly suggested that some frog species, such as the American bullfrog *Rana catesbeiana*, may in fact act as carriers of the fungus, with *Bd* infecting tadpoles and adults at persistent low levels without killing them (e.g., Garner *et al*., 2006; Skerratt *et al.*, 2007; Schloegel *et al*., 2010).

As for caudates, this group has been understudied with regard to *Bd* susceptibility, although newts and salamanders may be able to persist with or clear *Bd* infections to a greater extent than do anurans (Davidson *et al*., 2003). Still, few reports mention a possible mediation of *Bd* in enigmatic salamander population declines (e.g., Rovito *et al*., 2009). Thus, while not fully understood, host susceptibility is highly variable among species but also within the same species (Voyles *et al*., 2011).

1.2.1.2. *Batrachochytrium salamandrivorans*

In 2013 a new chytrid was described that was involved in the decline of fire salamanders (*Salamandra salamandra*) in the Netherlands, the *Bsal* (salamander chytrid). This fungus is another causative agent of chytridiomycosis that invades the skin and causes rapid death of its host, making it the second known chytrid that parasitizes vertebrates, particularly amphibians (Martel *et al*., 2013). Experimentally infected individuals have identical skin lesions to those seen in salamanders found dead during the decline (Martel *et al*., 2013; Spitzen-van der Sluijs *et al*., 2013). The pathogen seems restricted to, but highly pathogenic for, caudates (Martel *et al*., 2014), while anuran species such as the common midwife toad appear to be resistant to infection (Martel *et al*., 2013). The new emerging fungus has likely originated and remained in coexistence with a clade of salamander hosts for millions of years in Asia; the authors of a recent study suggest that it has recently been introduced into

some naïve European amphibian populations as a result of globalization and lack of biosecurity (Martel *et al*., 2014).

1.2.2. Ranaviroses

Although ranaviruses have been known for half a century (Granoff, 1966), their recognition as emerging pathogens is relatively recent. This may be a result of an increasing association with morbidity and mortality among animals from different taxonomic classes, but also of an increasing awareness of the disease and of wild population surveillance (Daszak *et al*., 1999; Chinchar & Waltzek, 2014). Either way, ranaviruses (belonging to family the Iridoviridae) have been identified as causative agents in several episodes of mass mortality in wild amphibian populations (Williams *et al*., 2005; Gray *et al*., 2009).

Ranavirus exposure can result in relatively mild viral infection that can be cleared even without developing clinical signs or generate asymptomatic hosts. However, more easily identifiable cases include the development of skin ulcerations and/ or systemic haemorrhages, ultimately resulting in death of the host (Cunningham *et al*., 1996; 2008; Robert *et al*., 2007). Mass mortality events associated with amphibian ranavirus have been described in Asia, Europe, North America and South America (Gray *et al*., 2009). Although also detected in Australia and Madagascar, episodes of mortality are unknown (Cullen & Owens, 2002; Kolby *et al*., 2015).

It has been suggested that the movements and translocation of fish, and possibly amphibians, may be responsible for driving the emergence of epizootics of this pathogen, thus posing additional threats to freshwater fish and herpetofauna (Hedrick & McDowell, 1995; Jancovich *et al*., 1997). For instance, the first outbreaks in Iberia coincided with the supposed introduction of pumpkinseed fish, this being suggested as a link between the two events (Soares *et al*., 2003). These *Ranavirus*-associated mortality events date back to 1998, where newts (both marbled newts *Triturus marmoratus* and Bosca's newts *Lissotriton boscai*) were found dead in the Peneda-Gerês National Park (Froufe *et al*., 1999; Soares *et al*., 2003). The individuals showed the typical signs of a ranavirose (with skin lesions and haemorrhages all over the body), but the phylogeny of this virus was never assessed, in order to understand its relationship to other Iberian *Ranavirus*.

A more recent study described a series of declines in two other areas of Spain (Galicia and Picos de Europa; Price *et al.*, 2014). Nevertheless, these new scenarios seem to contradict the general trend, showing a collapse at the level of amphibian host communities. In fact, most of the *Ranavirus* infections have been associated with epidemics that may reoccur annually, but host populations appear to persist (Brunner *et al*., 2004;

Greer *et al*., 2005). However, Price *et al*. (2014)'s new data are questioning that paradigm, raising concerns about host population persistence in the face of such catastrophic events. Theory predicts that while the host populations are unlikely to go extinct solely due to influence of the pathogen, their numbers may drop down to low levels, making specific species much more susceptible to stochastic events (Lafferty & Gerber, 2002).

With relatively few exceptions, most of the literature on die-offs reports single events or mortality in captive populations with no analyses of host population trends and dynamics in the face of the emerging threat. Thus, the need for long-term monitoring studies that track local amphibian populations over time has been repeatedly emphasised by several authors in order to quantify the impacts of (recurring) die-offs on recruitment and population size (e.g., Hoverman *et al*., 2012; Gray & Miller, 2013).

1.2.3. Host specificity of amphibian pathogens

Most pathogens (both human and wildlife) are capable of infecting more than one host species, and yet often display different levels of infectivity to other host species (Woolhouse *et al*., 2001). While a single-host pathogen can evolve to become highly detrimental to the host immune system, in a multi-host pathogen the favourable adaptation to one host species may be less effective to another host species (Elena *et al.* 2009). This is referred to as the species barrier (Combes & Théron, 2000). It is thus interesting to note that the simpler of single-host systems have generally raised more interest than generalist pathogens, despite the broad presence across systems of the later (Woolhouse *et al*., 2001).

Significant advances have been made to incorporate ecological concepts into research on wildlife pathogens and diseases (Tompkins *et al*., 2011) showing, for instance, that pathogens/ parasites have the ability to change the outcome of interspecific interactions such as predation or competition (e.g., Thomas *et al*., 1998; Holt & Dobson, 2006; Wood *et al*., 2007). However, natural history and behavioural principles have been largely ignored, particularly the way the host behaviour may mediate the impact of a pathogen.

A more comprehensive integration of host and pathogen life histories (Barrett *et al*., 2008) into theoretical and empirical studies would thus improve our knowledge on host-pathogen dynamics (Vander Wal *et al*., 2014). This approach would, for example, parameterize disease models with more robust data. Despite increasing interest in wildlife diseases, little attention has been also given to host-pathogen interactions in population viability analyses (Gerber *et al*., 2005). Overall, understanding cross-scale interactions in a

disease context may offer key insights into bigger picture questions such as when pathogens can cause species extinctions or the taxa most likely to be affected.

1.3. Aims

Quantifying the impacts and dynamics of diseases in wildlife populations is difficult, and becomes harder when working with multi-host pathogens. Understanding the properties of the host–parasite relationship in the wild is thus key to evaluating the impact of these pathogens on (multi-host) communities.

The main objective of this thesis is to develop a robust framework for modelling and understanding the spread and impacts of two emerging agents (*Bd* and *Ranavirus*) on amphibian assemblages. This main objective is broken down into several sub-goals:

1) Investigate the differential impact of two emerging pathogens on an amphibian diversity hot-spot in Europe, providing a record of population trends tracked over time following the outbreaks of disease [Chapters II, III and IV];

2) Analyse the dynamics of a generalist pathogen in a multi-host system with species with different susceptibilities, assessing the cost of exposure [Chapter III];

3) Investigate how the phenology of a host species mediates the impact of a generalist pathogen that causes sex-specific patterns of mortality [Chapter V];

4) Evaluate the risk of extinction of a population under different scenarios of sex-biased mortality mediated by a virulent pathogen; understand how differences in phenology will affect host population persistence in the context of infectious diseases [Chapter V].

The complex interaction between host, pathogen and the environment poses a challenge for conserving wild populations and assemblages. This study provides an innovative basis for enhancing environmental management and monitoring of these and other emergent diseases in the future.

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Chapter II

Evidence of chytrid-mediated population declines in common midwife toad in Serra da Estrela, Portugal *

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Evidence of chytrid-mediated population declines in common midwife toad in Serra da Estrela, Portugal

Abstract. The emergence of a novel infectious disease, chytridiomycosis, is now widely recognized as a major cause of amphibian declines and biodiversity loss across local and global scales. Amphibian mortalities caused by the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*) were first recorded in Iberia, Europe over a decade ago. In August 2009, hundreds of post-metamorphic common midwife toads (*Alytes obstetricans*) were found dead in the water and margins of a pond in the Serra da Estrela Natural Park, north-central Portugal. Histological and genetic analyses confirmed their infection with *Bd*. Given the likelihood of a new outbreak of chytridiomycosis, we evaluated the possible impacts of this disease on populations of *A. obstetricans* within the Park by conducting field surveys during 2010 and 2011. We compared the present distribution and abundance of *A. obstetricans* with historical records, and quantified the present prevalence and intensity of infection by *Bd*. Results showed that (1) *A. obstetricans* disappeared from 67% of the 1 x 1 km squares where it was recorded in the past, (2) breeding is currently limited to 16% of the confirmed breeding sites in the past, and that (3) larvae are now less abundant, as well as are highly infected by *Bd* in the remaining sites. These effects were most pronounced at altitudes above 1200 m. Our findings suggest that an outbreak of chytridiomycosis is responsible for the rapid decline of *A. obstetricans* in Serra da Estrela, and we believe that urgent conservation measures are needed to prevent local extinction of the species.

2.1. Introduction

Amphibian species are declining at an alarming rate on a global scale and are currently considered among the most threatened groups of vertebrates, with up to one-third of species threatened by extinction (Stuart *et al*., 2004). The global decline of amphibians is caused by multiple factors, the most prominent being habitat loss, invasion of nonnative species, overexploitation and outbreaks of infectious diseases. It is now widely recognized that the emergence of a novel infectious disease, chytridiomycosis, is driving rapid amphibian declines and biodiversity loss at the global scale (Berger *et al*., 1998; Stuart *et al*., 2004). The most severe outbreaks of chytridiomycosis have been described from the American Neotropics where the disease is reported to have been responsible for the extinction of about 67% (110 species) of *Atelopus* spp. frogs all across the distribution range of the genus (La Marca *et al*., 2005) and to have caused the rapid loss of amphibian biodiversity across eight families of frogs and salamanders at El Cope, Panama (Lips *et al*., 2006). However, severe impacts of chytridiomycosis have also been recorded across diverse tropical, temperate and montane environments, including the Caribbean, South America (particularly the Andean region), Australia, North America and Europe (Fisher *et al*., Walker, 2009b; Catenazzi *et al*., 2011).

The aquatic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is the etiological agent of chytridiomycosis (Fisher *et al.*, 2009a) and is known to infect over 508 species of amphibians (http://www.bd-maps.net/). The pathogen produces two key lesions: the loss of sections of keratinized mouthparts in amphibian larvae (Fellers *et al*., 2001; Knapp & Morgan, 2006), and the proliferation of keratinaceous cells and hyperkeratosis in postmethamorphic individuals (Berger *et al*., 1998; Kilpatrick *et al*., Daszak, 2010). The final mechanism by which *Bd* causes morbidity and mortality in amphibians appears to be the disruption of skin integrity and cutaneous function, leading to a subsequent loss of homeostasis (Voyles *et al*., 2009).

In Europe, *Bd* was first recorded causing mass amphibian die-offs during the summer of 1997 (Bosch *et al*., 2001). Southern European countries host most of the European amphibian biodiversity, and the Iberian Peninsula in particular shows a high number of endemic species (Pleguezuelos *et al*., 2002; Loureiro *et al*., 2008). Although it is imperative to understand the impact of chytridiomycosis in Iberia, mass die-offs caused by *Bd* are poorly documented except for some intensively surveyed localities in Spain (Garner *et al*., 2005) where amphibian species are apparently undergoing *Bd*-associated die-offs (Bosch *et al*., 2001; Bosch & Martínez-Solano, 2006; Walker *et al*., 2010). The best

documented case of an amphibian decline occurred at the Peñalara Natural Park (Sierra de Guadarrama, Central Spain) between 1997 and 1999 (Bosch *et al*., 2001). During this period, thousands of post-metamorphic common midwife toads (*Alytes obstetricans*) were found dead in several ponds within the park. By 1999, *A. obstetricans* had disappeared from 86% of the ponds it occupied just a few years before, and estimated larval density decreased sharply in three of the five ponds that still maintained larvae (Bosch *et al*., 2001). In addition, salamanders (*Salamandra salamandra*) and toads (*Bufo bufo* and *Epidalea calamita*) subsequently experienced population declines across this area (Bosch & Martínez-Solano, 2006, pers. obs.). Mapping the distribution of *Bd* across Iberia in *A. obstetricans* showed that infection by *Bd* was widespread in 31/126 sites (*n* = 3016 sampled individuals); however, only two other fatal outbreaks of chytridiomycosis were recorded in *A. obstetricans* populations in the Western Pyrenees and the Cantabrian Mountains (Walker *et al.*, 2010). Mapping the distribution of *Bd* emphasized the context-dependent nature of fatal chytridiomycosis as all occurrences of the disease were found at altitudes above 1600 m (Walker *et al*., 2010), confirming the previously noted relationship between cooler temperatures and chytridiomycosis (Berger *et al*., 2004; Drew *et al*., 2006; Kriger & Hero, 2007).

During August 2009, hundreds of post-metamorphic *A. obstetricans* were found dead either in water or dried on the surroundings of a pond (Lagoa do Covão das Quelhas; 1810 m a.s.l.; Fig. 1) in the Serra da Estrela Natural Park (PNSE), Portugal. Clear similarities between the observed pattern of mortality and the mass die-offs of the same species in the Peñalara Natural Park (Bosch *et al*., 2001) and the Pyrenees (Walker *et al*., 2010) raised the possibility of a new outbreak of chytridiomycosis. Dead post-metamorphic individuals were collected, and diagnostic analyses were undertaken at the Museo Nacional de Ciencias Naturales (Madrid, Spain) confirmed that the animals were highly infected with *Bd* and that mortality was likely caused by the fungus owing to the high susceptibility of *A. obstetricans* to this infection (Bosch *et al*., 2001; Walker *et al*., 2010).

To investigate the possible impact of the disease on *A. obstetricans* populations of the PNSE, we conducted a field survey during 2010 and 2011 aimed at (1) comparing the current distribution and abundance of *A. obstetricans* against historical records available from studies conducted during the 1990s, and (2) quantifying the prevalence and intensity of *Bd* infection in the larval stages of *A. obstetricans*.

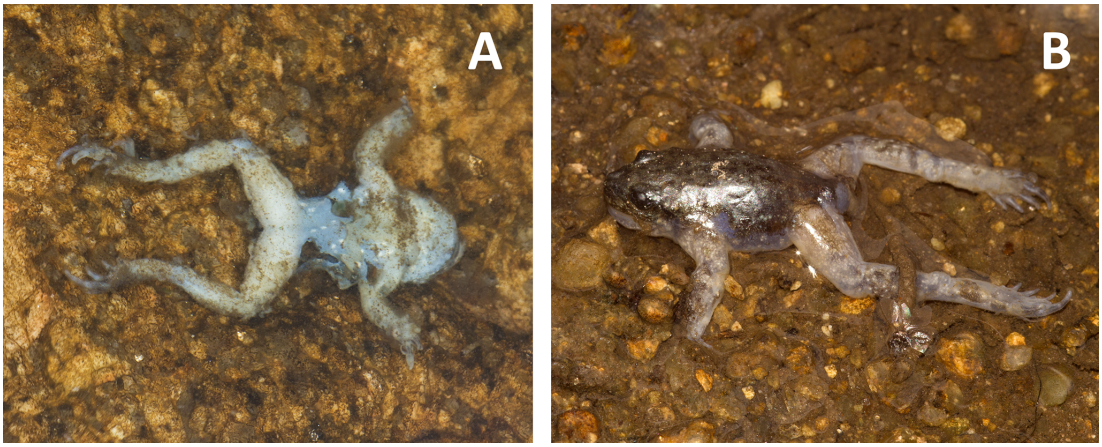

Figure 1. Post-metamorphic *Alytes obstetricans* individuals found dead (**A**) in water and (**B**) drying on the margins of a pond in Serra da Estrela Natural Park (Portugal).

2.2. Methods

2.2.1. Geography and biology of *A. obstetricans*

Serra da Estrela holds the highest summit (1993 m at Torre Plateau) of continental Portugal, and it is one of the highest mountains in the Iberian Sistema Central. The mountain range is located in north-central Portugal (40° 20'N, 7° 35'W) and integrates the PNSE. The Planalto Superior (area above 1400 m and up to the summit) presents stair-like plateaus dissected by glacial sculpted valleys (Daveau, 1971; Mora, Vieira & Alcoforado, 2001) and is characterized by a rich network of freshwater environments that is fed by high precipitation and is set within a landscape composed mainly of mat grass swards, shrub lands, rocky outcrops and patchy natural woodland (Malkmus, 1985; Jansen, 1997). Freshwater environments comprise many temporary and permanent ponds, and deep oligotrophic lagoons (not exceeding 10 ha) of glacial origin, swamps, bogs, temporary ponds, a dense system of springs, brooks and rivers, and artificial tanks, particularly in low altitude areas (Costa *et al*., 2004). The climate is temperate-Mediterranean with Atlantic influences and shows dry and warm summers and a wet season from October to May with frequent snowfall at higher altitudes (Daveau *et al*., 1977; Andrade *et al*., 1992; Jansen & Sequeira, 1999). Mean annual temperature is 3-4°C at the highest altitudes (Vieira & Mora, 1998), and mean annual rainfall reaches its maximum value at the Planalto Superior (exceeding 2500 mm) and a minimum in the northwest region (about 900-1000 mm) (Daveau *et al*., 1977; Jansen, 2002).

The Serra da Estrela supports the richest regional biodiversity in Portugal. It is one of the Iberian Peninsula biodiversity hotspots and harbors many endemic taxa, rare and

endangered species (e.g. Teles, 1970; Costa *et al*., 1998, 2003; Moreira *et al*., 1999). It harbors 13 species of amphibians, namely five species of newts and salamanders, and eight species of frogs and toads (Malkmus, 1985; Brito-e-Abreu *et al*., 1994; Godinho *et al*., 1996; Lesparre & Crespo, 2008; Loureiro *et al*., 2008).

According to a study carried out at the Planalto Superior by Brito-e-Abreu *et al*. (1994), *A. obstetricans* occupied all levels of altitude and was remarkably abundant when compared with population abundances at other regions of the country. During the 1990s and up to at least 2004-2006 (authors' pers. obs.), late-Spring choruses comprising many males were a distinctive feature of the landscape at dusk both at the Planalto Superior and its outskirts. The reproductive period of the species at the Planalto Superior occurs between May and August, with the maximum number of larvae being observed in August (Brito-e-Abreu *et al*., 1994). Frequently, more than one season is needed for completion of the larval development so that larvae from previous years can often be found coexisting alongside recently hatched individuals (Almeida *et al*., 2001).

In Portugal, *A. obstetricans* shows a notable degree of geographic and genetic differentiation. Populations across the north and centre of the country are not panmictic, and the populations from Serra da Estrela, in particular, were genetically differentiated from other geographically close populations (e.g. Serra da Malacata) (Rosa, 1995; Fonseca, 1999). The species is evaluated by the Red List as 'Least Concern' both in Portugal (Cabral *et al*., 2005) and globally (IUCN, 2011), but these conservation status do not yet reflect the high susceptibility of the species to chytridiomycosis and the recorded dramatic declines owing to this disease.

2.2.2. Amphibian surveys

In April, June and September 2010, and in May and August 2011, we carried out a comprehensive survey of all the aquatic environments previously identified as suitable breeding sites of *Alytes*, located in 38 sampling sites (1 x 1 km UTM grid squares) of the PNSE. The selection of the areas was based on authors' experience and extensive knowledge of the terrain, and previous studies (see Brito-e-Abreu *et al*., 1994; Godinho *et al*., 1996). Thirty of these UTM squares had been sampled in the 1990s as part of the characterization of the communities of amphibians and reptiles of the PNSE (Brito-e-Abreu *et al*., 1994; Godinho *et al*., 1996). In most of the cases, sites had been visited by J. Conde, F. Martins and P. Moreira with high regularity prior to 2009 and had been confirmed to hold breeding populations of *A. obstetricans*. For 19 UTM squares, data are also available on past abundance of larvae and adults (Brito-e-Abreu *et al*., 1994). In order to compare

the past and present distributions and abundances of *A. obstetricans*, we replicated the surveying methods previously employed (Brito-e-Abreu *et al*., 1994; Godinho *et al*., 1996). Surveys were planned to cover several months in 2 consecutive years in order to maximize sampling effort while reducing the likelihood of not detecting individuals because of the effects of seasonality and the natural population fluctuations (Pechmann *et al*., 1991; Skelly *et al*., 2003; Pellet & Schmidt, 2005). We surveyed slow-moving streams, natural and artificial lagoons, and stagnant permanent ponds and pools for larvae using hand nets. Furthermore, we surveyed terrestrial environments through active searching of adults under rocks. Time was roughly proportional to the area surveyed, varying between 1 and 4 hours: maximum time spent corresponded to the 1 x 1 km square grid area. This difference results from the heterogeneity of the landscape in terms of presence and area of water bodies within the surveyed grids. We recorded the location (using GPS), life stage, sex and health status (with special attention to possible evidences of the presence or absence of clinical signs of *Bd* such as lethargy, lack of righting reflex, reddening or sloughing of skin; Nichols *et al*., 2001) of all the observed individuals. Dead specimens were counted and collected, and preserved in 70% ethanol for subsequent scanning electron microscopy (SEM) and histological analysis (Berger *et al*., 1998; Berger, Speare & Kent, 1999). The quantification of the prevalence and intensity of *Bd* infection was undertaken by swabbing the skin of post-metamorphic animals and of the keratinized mouthparts of larval stages according to protocols described by Boyle *et al*. (2004) and Hyatt *et al*. (2007).

To prevent spreading the disease, disposable latex gloves were used to handle animals. Other field equipment used during the surveys (including hiking boots) was exclusively used in the study area and was periodically immersed in a 1% solution of Virkon® (Antec International ltd., Sudbury, Suffolk, UK) before and after being used in order to avert potential cross-contamination according to the protocol suggested by Speare *et al*. (2004).

2.2.3. Detection of chytridiomycosis

Swabs were assessed for the presence of *Bd* using quantitative, real-time polymerase chain reaction (qPCR). All analyses were performed in accordance to the protocol by Boyle *et al*. (2004) using the qPCR experiments in 96-well plates and analyzing the (1 : 10) DNA extract dilutions of each sample in duplicate. Standards of known zoospore concentrations and negative controls were included in each plate Samples were considered positive when a sigmoidal amplification occurred in both duplicates PCR reactions and a threshold quantity

greater than 0.1 genomic equivalents (GEs) between the two runs was obtained. The threshold value has been adopted in all of our publications to date.

2.2.4. Data analyses

For each sampling site, we determined the presence/absence of *A. obstetricans* and quantified the abundances of larvae and post-metamorphic individuals (including recently metamorphosed individuals, juveniles and adults). Data about the present status of *A. obstetricans* was compared with data collected prior to 2009. However, for some UTM squares sampled in the past, information is limited to presence/ absence of *A. obstetricans*. Considering both past and present data, we created categories of relative abundance of larvae and post-metamorphic individuals per 1 x 1 km UTM square. Categories were obtained based on sampling effort (individuals/time) per 1 x 1 km grid square: larvae: *not detected* 0, *low* between 1 and 100, *medium* between 101 and 500, and *high* > 500; post-metamorphics: *detected* and *not detected*. We considered the maximum number of animals observed across all sites that were surveyed on multiple occasions during 2010 and 2011. We considered *A. obstetricans* to be currently not detected from a UTM square when we did not observe any individual or hear any male call during our surveys of 2010 and 2011 (in the case of adult individuals). Data from the past and present were mapped using ESRI's ArcMap 10.

Matrices of presence/absence information of larvae and post-metamorphics in the past and present (larvae past vs. present and post-metamorphics past vs. present) according to UTM squares were compared by performing the chisquare test. This analysis included only the 30 squares that were surveyed both in the 1990s and 2010-2011, and it was used to compare binary data sets. We tested for the effect of altitude on infection prevalence and infection load by *Bd* by using a binomial logistic regression. Moreover, we tested whether outbreak of fatal chytridiomycosis causing extirpation (absence of individuals in sites where the species was recorded in the past) or sharp reduction of larvae density were related to altitude using an exact logistic regression (Hirji *et al*., 1987). All the statistical analyses were performed with SPSS statistics v.19.0 (for Windows) (IBM Corp., Armonk, NY, USA). Prevalence of *Bd*-infected animals was calculated by dividing the number of individuals positive for *Bd* by the corresponding number of individuals that we swabbed. Ninety-five per cent binomial confidence intervals (CIs) were calculated for prevalences by the Clopper–Pearson method. These analyses were performed using QP 3.0 software (Quantitative Parasitology 3.0, Budapest; Reiczigel & Rózsa, 2005) that gives priority to biological realism (Rózsa *et al*., 2000).

Quantification of chytrid zoospores on infected individuals is given as the mean value of zoospore equivalents detected (GE) in the two replicates of the PCR analysis. We used this number as an index of the intensity of an individual's infection.

2.3. Results

In 2009, we analyzed nine dead post-metamorphic individuals (by histology, SEM and qPCR), and between 2010 and 2011, we sampled 217 individuals of *A. obstetricans* (*n* = 188 larvae, *n* = 29 post-metamorphics; by qPCR) from across 38 1 x 1 UTM grid squares. We found *A. obstetricans* in 13 of the 38 1 x 1 UTM squares of the PNSE, but larvae were observed in just 10 UTM squares (Fig. 2; Table 1). Thirty of our 38 UTM squares were occupied by the species in the 1990s (Brito-e-Abreu *et al*., 1994; Godinho *et al*., 1996; Fig. 2) and, in some cases, shortly before 2009 (pers. obs.). This decrease in the distribution of the species since the 1990s represents the extirpation of populations from 20 (about 67%) of the UTM squares where it was once confirmed to occur. Furthermore, larvae were found to persist in only six (21%) of the 29 ponds and lagoons that were confirmed as suitable breeding sites in the past. Among these sites, only Lagoa dos Cântaros, a medium-sized natural lagoon, is located above 1200 m. The remnant four sites (Tanque de Folgosinho, Charco de Sazes, Tanque dos viveiros florestais de Sazes and Tanque do Alvoco) are irrigation and fire-fighting water tanks located below 1000 m. Larval density has decreased sharply in three of the four lagoons that still maintained larvae above 1200 m in 2010 and 2011 when compared with estimations from the past. A chi-square test showed a significant difference between past and present presence per UTM square for both groups: χ^2 = 37.3 (*d.f.* = 1, $p < 0.001$) for larvae and χ^2 = 30.0 (*d.f.* = 1, $p < 0.001$) for post-metamorphics.

Although we did not record any larvae among the aquatic environments surveyed in the UTM square 617E 4466N during 2010 and 2011, we observed a couple in amplexus in 2010 at one of the ponds (Salgadeiras). A juvenile *A. obstetricans* was also seen in the UTM squares 617E 4471N and 616E 4471N (Barragem do Lagoacho) in 2010, thus indicating successful reproduction at least in 2009.

With the exception of five larvae from Ribeira de Leandres (see Table 1) that had reduced denticles, none of the larvae showed morphological lesions typically associated with chytridiomycosis. All larvae showed normal swimming behavior and appeared to be healthy. Most of the dead post-metamorphic individuals showed no consistent gross

lesions or any evident signs of chytridiomycosis, except for nine individuals that were too decomposed to be assessed for signs of disease.

Table 1. Prevalence of the fungus *Batrachochytridium dendrobatidis* in common midwife toad (*Alytes obstetricans*) larval individuals at Serra da Estrela Natural Park during 2010/ 2011. Prevalence includes 95% confidence intervals (CI).

Name of the water point	UTM Coordinates (zone 29 T)		Elevation (m)	Habitat	*n*	Prevalence 95% CI (range)	Infection load [mean GE* (min-max)]
	x (m E)	y (m N)					
Tanque da Ribeira de Leandres	626585.42	4472678.38	651	tank	18	0.39 (0.1729-0.6426)	4439.6 (38.7-13667.5)
Charco de Sazes	608488.24	4466769.72	780	pond	20	0.15 (0.0320-0.3790)	89.4 (10.0-200.4)
Tanque do Alvoco	611402.16	4461861.85	861	tank	3	0.33 (0.0084-0.9058)	79379.3 (79379.3-79379.3)
Tanque dos viveiros florestais de Sazes	609168.04	4466772.82	985	tank	20	0.60 (0.3605-0.8089)	5242.9 (2.4-13621.2)
Tanque de Folgosinho	624581.89	4483586.33	1079	tank	21	0.33 (0.1458-0.5697)	2456.5 (22.3-10326.2)
Charco recreativo #	610565.15	4466869.68	1263	pond	21	0.90 (0.6962-0.9883)	10173.8 (5.5-49919.2)
Lagoa dos Cântaros	619550.18	4465996.29	1646	lagoon	16	1.00 (0.7940-1.0000)	450.3 (80.01198.4)
Represa da Torre	618178.00	4464900.67	1955	mill-pond	21	1.00 (0.8389-1.0000)	1101.4 (10.0-3210.0)

* mean number of zoospore equivalents detected on infected larvae sampled at a site

this pond did not exist back in 1990 decade, being created upon the road N338 opening in 2006. Unfortunately, it was silted after a fire following 2010 sampling and no larvae were recorded in 2011

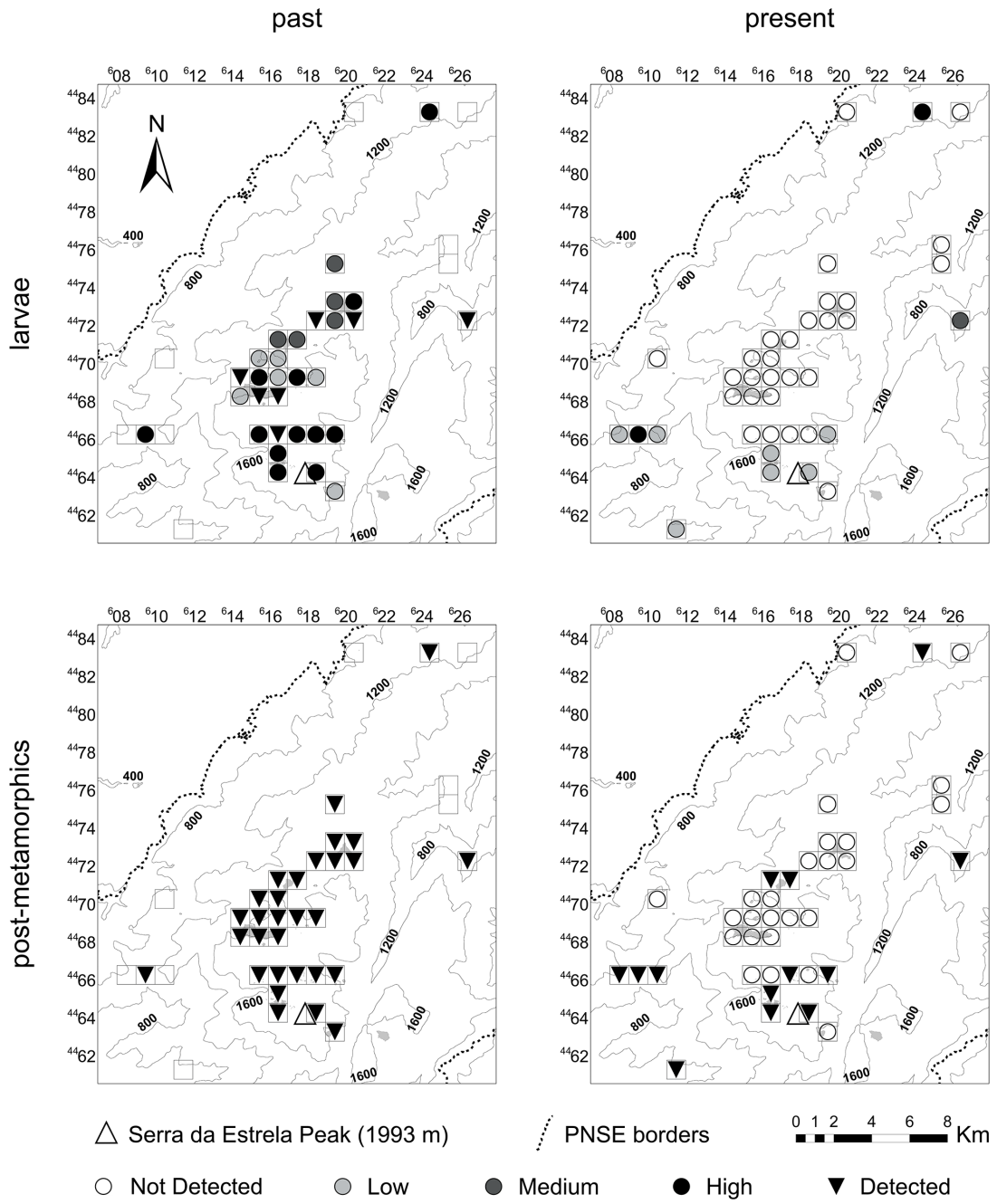

Figure 2. Relative abundance of common midwife toad (*Alytes obstetricans*) larvae and post-metamorphics at Serra da Estrela Natural Park (PNSE). *Past* maps summarize data prior to 2009, and *present* maps show the result of surveys carried out during 2010/2011. The relative abundance is represented by a color gradation. Data from some grid squares are in some cases restricted to presence/absence. Axes show 1 x 1 km UTM coordinates.

SEM examination of skin of dead post-metamorphic specimens revealed evidence of fungal infection by marked roughening of the skin with small openings (crater-like structures) on the epidermis. As referenced elsewhere (e.g. Berger *et al*., 1998; Bosch *et al*.,

2001; Bosch & Martínez-Solano, 2006), these prominent tubular structures are likely produced by fungal sporangia, allowing the dispersal of the zoospores from toads' skin.

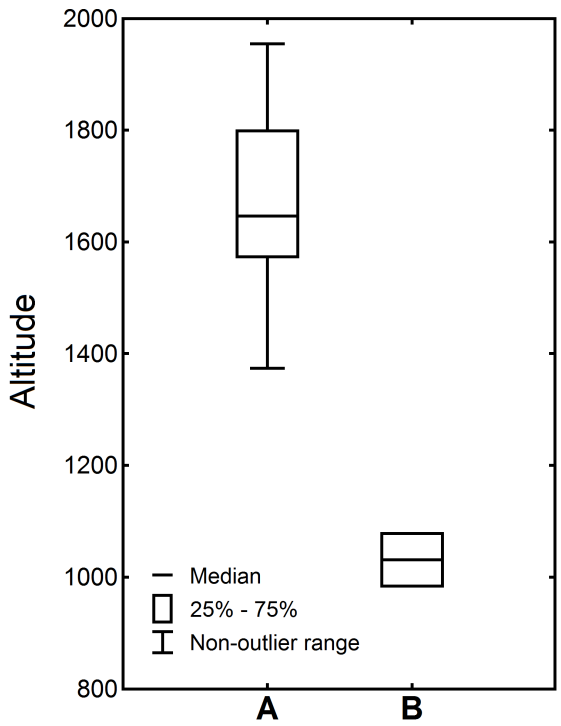

Figure 3. Box-plots displaying the altitude (m) associated with known outbreak of fatal chytridiomycosis causing extirpation or sharp reduction of larvae density (**A**) and at localities from which *Batrachochytrium dendrobatidis*-infected amphibians occur, although no sharp reduction has been recorded (**B**). The difference is significant at 5%.

Prevalence of *Bd* infection among the eight sites where larval individuals were swabbed ranged between 15 and 100%, with a median of 49% and an interquartile range from 33.3 to 92.9% (Table 1). Prevalence of *Bd* was significantly correlated with altitude (*Wald* = 27.71, *d.f.* = 1; $p < 0.001$). Moreover, localities with known outbreaks of fatal chytridiomycosis were significantly associated with altitude (*Model score* = 23.72483, $p < 0.0001$; Fig. 3). Considering only the sites located above 1200 m and thus including the whole Planalto Superior, the prevalence of infection among the four pools and lagoons where larvae were found ranged from 90 to 100%, with a median of 100% (n = 3). Two lagoons showed a prevalence of infection of 100%. In contrast, for lower altitude sites, prevalence ranged between 15 and 60%, with a median of 33.3% and an interquartile range of 5.56 (n = 5) (Table 1). In contrast with the prevalence pattern, *Bd* infection load showed no significant association with altitude (r_s = -0.16; $p > 0.05$).

2.4. Discussion

We describe here a novel episode of mass mortality in post-metamorphic individuals of *A. obstetricans* from the Portuguese Serra da Estrela. According to Brito-e-Abreu *et al*. (1994), the Planalto Superior region used to contain abundant populations of many anuran species, including the midwife toad. Choruses of calling males used to be readily heard during the

breeding season, and ponds supported high densities of larvae. In addition to the reduced number of sites where larvae were sighted, the lack of direct observations of breeding adult toads suggest that just a few pairs managed to reproduce in the aquatic environments that we surveyed during 2010 and 2011. Our findings point to an outbreak of chytridiomycosis likely being responsible for the population decline and observed disappearance of this species. This represents the first report of amphibian population decline associated with the presence of *Bd* in Portugal. The circumstances that we describe are very similar to those described by Bosch *et al*. (2001) for the Peñalara Natural Park population, with rapid declines being observed in populations living at high altitudes in well-preserved habitats, and the extreme nature of the events that we document waives the possibility of natural population fluctuations. Given that the episodes of mass mortality have not been noticed in this region prior to 2009, it appears that we are witnessing a recent introduction of *Bd* into a previously uninfected region perhaps mediated by associated climatic factors, as has been previously hypothesized (Bosch *et al*., 2007).

Data from other infected sites in the Iberian Peninsula (the Sierra de Guadarrama, the Pyrenees and the Cantabrian Mountains) show a median prevalence of infection ranging between 12 and 90% (Walker *et al*., 2010). Particularly in Peñalara Natural Park, prevalence is 100% above 1600 m (Bosch *et al*., 2001; Walker *et al*., 2010), similar to that which we observe at high-altitude sites in Serra da Estrela (100%). On the other hand, *Bd* infection load across the different ponds did not show a pattern with altitude. This can be because no association actually exists or simply because of the use of average GE values that obscure the actual pattern. *Bd* infection load values are often highly non-normal because of the presence of extreme values within the same site influenced by different biotic and abiotic variables (see, e.g. Rödder *et al*., 2008; Stockwell, Clulow & Mahony, 2010; Tobler & Schmidt, 2010). Therefore, averaging GE values within a population may produce highly misleading results.

Several hypotheses could explain why species of *Alytes* are more susceptible to *Bd* than other sympatric amphibian species. Species-specific antimicrobial skin peptides (AMPs) are key determinants of survival (Woodhams *et al*., 2007); however, it is not known whether *A. obstetricans* produces anti-*Bd* AMPs and whether these are less effective when compared against *Bd*-resistant species, such as *Rana temporaria*. Bosch *et al*. (2001) suggested a combined action of relatively small clutch sizes and extended larval period caused by cold temperatures during winter leading to a higher probability of contact with the *Bd* zoospores and higher infectious burdens; this overwintering behavior has been shown to affect survival in the Sierra Mountain yellow-legged frogs *Rana muscosa* (Briggs,

Knapp & Vredenburg, 2010). However, these observations raise a yet unanswered question: What factors can explain the wide range in prevalence of *Bd* in sites with similar elevation? For example, Tanque de Folgosinho at 1079 m a.s.l. (33%) versus Tanque dos viveiros florestais de Sazes at 985 ma.s.l. (60%). Is larval density the key? Can different prevalences of *Bd* be explained by presence/absence of other amphibian species or even non-amphibian vectors/reservoirs? Are the infecting genotypes of *Bd* all similarly virulent (Fisher *et al*., 2009b; Farrer *et al*., 2011)? Are the populations of *A. obstetricans* all equally susceptible to infection? Do limnological factors matter? Does the presence of predators of larvae play a role? Amphibian larvae often experience elevated levels of stress when exposed to high interspecific and intraspecific densities and/or predatory risks (Skelly, 1992; Carey *et al*., 2003), which can lead to an exacerbated vulnerability to the infectious fungus (Parris & Beaudoin, 2004; Garner *et al*., 2009; Savage & Zamudio, 2011). The reasons could be environmental or even geographical, but genetic differences between *Alytes* populations are likely a source of at least part of this variation (Tobler & Schmidt, 2010; Savage & Zamudio, 2011). However, given the wide CIs for prevalence, we cannot discard the possibility that difference in prevalence among populations is due to sampling variation. Clearly, there are many variables that may affect the host/ pathogen dynamic, tipping it in favor of the frogs or the pathogen, and further research needs to be undertaken in order to determine the outcome of this host/pathogen dynamic following the introduction of *Bd*.

An anecdotal observation increased our concern during the summer of 2010 when monitoring the Salgadeiras revealed two individuals of Perez's frog (*Pelophylax perezi*) found dead and manifesting a high burden of *Bd*. Other anuran species, such as the common toad (*Bufo bufo*), the Natterjack (*Epidalea calamita*), and urodeles like the fire salamander (*Salamandra salamandra*), the Bosca's newt (*Lissotriton boscai*) and the marbled newt (*Triturus marmoratus*), share these higher altitude mountain areas with the midwife toads (Lesparre & Crespo, 2008). However, no declines have been detected in these populations. Although the effect of chytridiomycosis on urodeles is still poorly understood, cases of infection in the wild have been documented at Peñalara where populations of *B. bufo* and *S. salamandra* have experienced a significant decline (Bosch & Martínez-Solano, 2006).

These new findings place high elevation amphibian populations and particularly the common midwife toad in a critical state of concern within this Natural Park. Our data suggest that *Bd* is still increasing its range within Iberia by invading uninfected populations. However, as yet, we have no clues as to the vector that introduced the infection. Owing to

the similarity of our study area with the Peñalara Natural Park, our findings suggest a near extirpation of *A. obstetricians*. Further mass mortality episodes above 1600 m may occur in the Serra da Estrela, and population viability analyses are urgently needed. More widely, the risk that chytridiomycosis manifests to amphibian communities across the Iberian Peninsula poses a crucial challenge for wildlife managers and raises an urgent need for targeted conservation strategies, such as captive-breeding and treatment protocols for infected amphibians.

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Chapter III

Differential host susceptibility to a globally emerged infectious agent: sub-lethal costs of a virulent pathogen

Differential host susceptibility to a globally emerged infectious agent: sub-lethal costs of a virulent pathogen

Abstract. Pathogens can have strong effects on individual hosts with a variety of adverse effects, eventually leading to disease and host mortality. Multi-host pathogens can infect more than one species and persist in a transmission cycle where the pathogen is maintained in several species. This seems to be the case for an amphibian pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). However, while some species seem to have disappeared completely following *Bd* outbreaks, other species persist without being (apparently) affected. In this study we aimed to understand the dynamics of *Bd* in a multi-host community, focusing on two amphibian species with different susceptibility. We address the role of a salamander host (*Salamandra salamandra*) as a reservoir, assessing the cost of exposure to the pathogen during the larval stage. Populations of *Bd*-sensitive *Alytes obstetricans* and apparently *Bd*-resistant *S. salamandra* were monitored between 2010 and 2014 in sites where *Bd* is known to be present. Our field surveys showed no significant fluctuation in the numbers of salamander larvae, contrasting with the decline of midwife toads. Both species share the same *Bd* infected freshwater habitats, but no *Bd* positive or *Bd*-mediated mortality of *S. salamandra* has ever been recorded in any of the monitored sites. Additionally, exposing *S. salamandra* larvae to a highly virulent strain of *Bd* showed that larvae are very resistant to the disease chytridiomycosis. After four weeks of repeated exposures, no infection was detected in most of the experimental groups, with the exception of the higher dose that resulted in 25% prevalence. However, sub-lethal costs were documented in exposed individuals, indicating that successful immune responses to *Bd* exposures come at a considerable trade-off. Costs were accrued in a dose dependent manner during the larval stage. However, in face of what has been reported in other systems, resistance seems not to be a consistent trait across fire salamanders.

3.1. Introduction

Pathogens can impact individual hosts with a variety of adverse effects. These include decreased growth and reduced development, and also life history and behavioural effects, increasing susceptibility to disease and ultimately resulting in indirect mortality (Dobson & Hudson, 1986; Kiesecker & Blaustein, 1999; Daszak *et al*., 2000; Poulin & Forbes, 2012).

The majority of known pathogens can infect more than one species (Woolhouse *et al*., 2001) and persist in a transmission cycle where the pathogen is maintained in multiple species (Cleaveland *et al*., 2001). While single-host pathogens tend to evolve an intermediate level of virulence enabling them to persist in their host species (Anderson & May, 1991; Woolhouse *et al*., 2001), multi-host pathogens can affect at least some of the hosts in a way such that they will not persist independently. Virulence evolution in multi-host pathogens is more complex since a pathogen can be highly virulent in one host while exhibiting low virulence in another (Woolhouse *et al*., 2001). Thus, these pathogens need reservoir hosts that make it possible for them to persist (Ashford, 1997), but occur outside the reservoir causing localized disease outbreaks (Rhodes & Anderson, 1996). Consequently, not specialising on a single host may render generalist pathogens less prone to extinction (Timms & Read, 1999).

In recent years, pathogenic fungi have become widely recognized as posing major threats to animal health, and are responsible for episodic mass mortalities, leading to declines in wildlife populations (Daszak *et al*., 2000; Smith *et al*., 2006; Fisher *et al*., 2012). Amphibian chytridiomycosis is a disease caused by the chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*) and is thought to be involved in massive declines of amphibians around the world (Berger *et al*., 1998; Fisher *et al*., 2009b). This highly pathogenic chytrid has been shown to infect over 500 species across all three groups of amphibians on all continents where they exist (Fisher *et al*., 2009b, 2012; Doherty-Bone *et al*., 2013; http://www.bd-maps.net/).

The effects of *Bd* on amphibian populations may vary between species, habitats, locations, and even in cases where potential hosts are sympatric (Stuart *et al.*, 2004; Kilpatrick *et al*., 2010; Walker *et al*., 2010; Searle *et al*., 2011b). While some species seem to have disappeared completely following *Bd* outbreaks (e.g. Lips *et al*., 2006), other species persist without (apparently) being affected, despite high levels of infection (Retallick *et al*., 2004; Briggs *et al*., 2005). At some extent this can be explained by ecological factors such as elevation (Walker *et al*., 2010). In some places, species appeared to be affected in a sequential way, supposedly according to their susceptibility. This is the case of the

amphibian community in Guadarrama (Central Spain), where *Bd* first caused *Alytes obstetricans* extirpation (Bosch *et al*., 2001), then influenced fire salamander (*Salamandra salamandra*) dynamics (Bosch & Martínez-Solano, 2006), and ultimately affected the common toad (*Bufo spinosus*; Bosch *et al*., 2014).

While susceptibility to the amphibian chytrid varies greatly between species, mortality from *B. dendrobatidis* infections appears to occur primarily in postmetamorphic individuals. However, exposure to *Bd* during the larval stage can still affect their fitness in terms of growth rates and other aspects of development (e.g., Parris & Baud, 2004; Garner *et al*., 2009).

Although correlation of *Bd* with loss of anuran populations has been well documented, with some species being identified as hyper susceptible to chytridiomicisis (e.g., *A. obstetricans*; Bosch *et al*., 2001; Rosa *et al*., 2013), the pathogenicity of the fungus to caudates is still poorly understood, particularly in Europe. Records of *Bd* affecting natural populations of European caudates are very scarce, despite the occurrence of the fungus within the range of many species. Indeed, terrestrial salamanders generally show an absence of *Bd* (e.g. Federici *et al*., 2008; Lötters *et al*., 2012; Pasmans *et al*., 2013; Baláž *et al*., 2014), as well as cave salamanders of the genus *Speleomantes* (Chiari *et al.*, 2013). After confirming absence of *Bd* from all European *Speleomantes*, Pasmans *et al*. (2013) even demonstrated a pronounced degree of resistance to chytridiomycosis due to highly efficient skin defences.

Understanding the properties of the host-parasite relationship is critical for long-term conservation (Murray *et al*., 2009). This line of research allows us to identify which species or populations are likely to be more susceptible to being affected (Bielby *et al*., 2008) or simply acting as a reservoir for a pathogen. In this study we aimed to understand the dynamics of an aquatic pathogen (*Bd*) in a multi-host community, focusing on two amphibian species with different susceptibility. We addressed the role of a widespread salamander host (*Salamandra salamandra*) as a reservoir of chytrid fungus, determining the minimum *Bd* load that allows the development of infection and assessing the cost of exposure to this pathogen during the larval stage.

3.2. Materials and methods

3.2.1. Study site

Located in north-central Portugal (40°20'N, 7°35'W), Serra da Estrela is a mid-high elevation system reaching 1993 m a.s.l. at the summit (Torre Plateau), and a part of the Iberian

Sistema Central. The mountain range integrates the Natural Park and is characterized by a rich network of permanent ponds and lakes of glacial origin (Malkmus, 1985; Jansen, 1997), swamps, bogs, temporary pools, and a dense system of springs, brooks and rivers (Costa *et al*., 2004). The climate is temperate-Mediterranean and influenced by the Atlantic, showing dry and warm summers and a wet season from October to May. Snowfall is frequent at higher altitudes where the mean annual temperature is 3-4°C (Daveau *et al*., 1977; Vieira & Mora, 1998).

Serra da Estrela is an Iberian Peninsula biodiversity hotspot, and presents many endemic, rare and endangered species (e.g., Teles, 1970; Costa *et al*., 1998, 2003; Moreira *et al*., 1999). It harbours 13 species of amphibians and among them the common midwife toad (*Alytes obstetricans*) and the fire salamander (*Salamandra salamandra*) (Godinho *et al*., 1996; Lesparre & Crespo, 2008; Loureiro *et al*., 2008).

3.2.2. Monitoring of *Alytes* and *Salamandra* populations

Five freshwater water sources (above ca 900 m) were monitored between 2010 and 2014 for *Alytes obstetricans* overwintering tadpoles and *Salamandra salamandra* larvae: Tanque dos Serviços Florestais de Sazes, Tanque de Folgosinho, Represa da Torre, Lagoa dos Cântaros and Lagoa do Covão das Quelhas (see Rosa *et al*., 2013 for further details on the sites). The selection of the areas (sampling units) was based on past data from Brito-e-Abreu *et al*. (1994) and data gathered during the study by Rosa *et al*. (2013), targeting ponds where both species were known to occur in the past (1990's decade) and *Bd* has been recorded. Sampling took place during springtime, where both species (larval stages) can be found at higher densities and sharing the freshwater habitats (GMR pers. obs.).

Abundances were determined using temporally and spatially replicated counts. In order to reduce the effect of variation in detection probability among years, each site was surveyed two to three times per year. The highest count per site (within the same year) was considered the peak abundance and later used in the analyses. The surveys duration was two hours per site, with two persons using dip-nets. To note that later in 2011, *Ranavirus* outbreaks were linked to mass mortality episodes of several amphibian species at Serra da Estrela, including *A. obstetricans* (Chapter IV). These yearly events prevented an analysis of the overall trend of the *Alytes* population mediated solely by chytridiomicosis.

To increase the sample size of *S. salamandra* (and thus the likelihood of detecting *Bd* in that species), we undertook a comprehensive survey of aquatic environments identified as suitable breeding sites for this species, with sampling taking place three to four times per year (spring time, summer and autumn/ winter season). This design also

allowed the gathering of data on infection prevalence in *A. obstetricans*. We surveyed slow-moving streams, natural and artificial lagoons, and stagnant permanent ponds and pools for larvae using hand nets. Adults were sampled opportunistically both during the day and night.

The quantification of the prevalence and intensity of *Bd* infection was done by swabbing (MW113, Medical Wire and Equipment Co) the mouthparts and/or the skin of animals targeting the keratinized tissues where chytrid grows (mouth and skin in larvae, and skin in adults of salamander; mouth in larvae and skin in post-metamorphs of midwife toads) according to protocols described by Boyle *et al*. (2004) and Hyatt *et al*. (2007).

To prevent spreading the disease, disposable vinyl gloves were used to handle animals. Other field equipment used during the surveys (including hiking boots) was exclusively used in the study area and was periodically immersed in a 1% solution of Virkon® (Antec International ltd., Sudbury, Suffolk, UK) before and after being used in order to avert potential cross-contamination according to the protocol suggested by Speare *et al*. (2004).

3.2.3. Artificial exposures and responses

Four gravid *S. salamandra* females were collected in late October, 2011 from a population in Sintra, Portugal (38°47'33.46"N, 9°23'48.88"W). Females were housed together and all gave birth in less than 24 h. After two weeks, all the larvae had developed their hind limbs (post-Harrison stage 46; Harrison, 1969) and were housed individually in 37x22x25 cm plastic containers filled with 3000 ml of aged tap water. Larvae were fed the same amount of bloodworms (five large individuals) every two days with water changes performed weekly. Experimental procedures were completed in a climate-controlled room. Water temperature was kept at 14°C ± 1°C with a 12:12h day/night light schedule.

Each salamander larva was weighed (ToWt) and snout-vent length (SVL, from tip of snout to anterior edge of vent) measured at the beginning and at the end of the experiment. Weight was measured by placing the larva on a zeroed balance (± 0.01 g) with absorbent paper for 10 s. Weight was calculated by deducting the weight of wet paper from the recorded weight (Browne *et al*., 2003). To obtain the SVL, the individuals were photographed against a measuring scale and length measured from images using ImageJ software (Rasband W.S., US National Institutes of Health, Bethesda, Maryland, USA). Body dimensions did not significantly differ among experimental groups at the start of the experiment ($p > 0.05$; see Data Analysis).

The experiment was divided into three phases: a *baseline*, a phase of *exposure* (where animals were expose to repeated doses of chytrid culture according to their treatment groups), and a *post-exposure* phase (where animals were just monitored for sub-lethal effects of the chytrid exposures). Salamander larvae were randomly assigned to one of the following treatments (each experimental group *n* = 8 larvae): repeated high (50 000 zoospores per 1 ml inoculum), medium (5 000 zoospores per 1 ml inoculum) and low (500 zoospores per 1 ml inoculum) doses of active *Bd* culture, and repeated sham exposures (*n* = 7; corresponding to the negative control: inoculum prepared following the same methods but without *Bd* in the broth). Acting as a positive control a further group of larvae was exposed to repeated substantially high doses (500 000 zoospores per 1 ml inoculum) of active *Bd* culture.

Upon every exposure, an additional inoculum was inoculated onto fresh media recipients to control and warrant that *Bd* zoospores were in fact active, viable and growing. We used a highly virulent isolate of the chytrid fungus (*Bd*GPL) generated from a dead *Alytes obstetricans* metamorph (isolate IA 2011). Zoospore concentration of *Bd* cultures was assayed prior to infection using a haemocytometer and by counting only visibly active zoospores. Fungal inoculum was prepared according to Garner *et al*. (2009).

During *exposure* phase larvae were inoculated every week over a period of one month for a total of four inoculations, with the exception of the positive control treatment which skipped the first exposure, being subjected to inoculation three times. We exposed each larva individually to *Bd* for 24 h in a separate plastic container filled with 200 ml aged tap water. After exposure, individuals were turned back to their containers. The experimental design mirrored the natural transmission by water, one of the possible modes of *Bd* transmission in natural environments (Pessier *et al.*, 1999). Determination of the infection status was made two weeks after the last exposure.

Activity and movement of the *S. salamandra* larvae were recorded every two days (except for larvae in the positive control group): behavioural observations started two days before the first exposure to *Bd* (*baseline*), continued throughout the exposure period (*n* = 23 days: *exposure*), with additional monitoring till the end of trial period (*n* = 6 days: *post-exposure*). Observations were made between 14:00 hr and 16:00 hr. In order to quantify activity, we recorded for each individual the number of movements and total time spent in motion over a one-minute period (termed "individual larva time of activity") using a stopwatch. Larvae were recorded as active when propelling themselves with the tail or walking on the bottom of the tank, and inactive when motionless, i.e. without displacing

from where they were (it includes turning of the head while remaining stationary, or floating without any movement). Observations were made 24 hours after the feeding.

In order to minimize the possibility of *Bd* transmission between *Bd* exposed or unexposed treatments, we always performed water changes and feeding of unexposed larvae before exposed larvae. Before starting the experiment we swabbed the adult females' whole body to ascertain *Bd* infection status and burden (all *Bd*-free). At the end of the experiment, we extracted mouthparts and one of the hindlimb feet of the larvae also for *Bd* screening. Tissue samples were stored in 70% ethanol until DNA extraction.

3.2.4. Detection of *Bd* and determination of the infection status

Swabs and tissue samples were analysed for the presence of *Bd* using quantitative (real-time) polymerase chain reaction techniques (qPCR) described by Boyle *et al*. (2004), and employing the changes described by Kriger *et al*. (2006). Extractions were diluted 1/10 before real time PCR amplification, performed in duplicate, and with *Bd* genomic equivalent (GE) standards of 100, 10, 1 and 0.1 GE. Samples were considered positive when a sigmoidal amplification occurred in both duplicate PCR reactions and a threshold quantity greater than 0.1 GEs between the two runs was obtained.

3.2.5. Data analysis

Differences in larval abundances for both species over the years were tested using a generalized linear model (GZLM), considering a Poisson distribution for the number of animals counted, and a log link function. We used "year" as a fixed effect and "pond/ site" as a random effect.

In the laboratory, to compare body measurements among experimental groups, we first normalized the *weight by cube-root transformation:* $ToWt^{1/3}$. Both ToWt and SVL were compared using one-way ANOVAs, confirming no significant differences between the experimental groups at the beginning of the experiment. Weight and length gain were also compared at the end of the experiment using analysis of covariance (ANCOVA) with starting weight/ length as covariates. When treatment effects were significant ($p < 0.05$) we proceeded with pairwise comparisons Fisher's LSD post hoc tests.

To examine differences in species behavioural responses among the *Bd* exposure treatments over the study period and between experimental groups in each of the three different phases of the study (*Baseline*, *Exposure* and *Post-exposure*), we used GLMMs with a Poisson distribution with a log link function for the number of movements and a normal distribution with an identity link function for the time of activity. "Treatment" and "phase"

were used as fixed effects and their interaction was also included in the model. Post-hoc pairwise contrasts were used to test for differences between all paired experimental treatments. All statistical analyses were carried out with software IBM SPSS 20.0 (IBM corp. Chicago, USA).

3.3. Results

3.3.1. Field surveys

We analysed the change in abundance of both species and found a highly significant effect of time for *Alytes obstetricans* ($F_{4, 16}$ = 8.826, p = 0.001) with this species experiencing a sharp decline in abundance, whereas *Salamandra salamandra* populations showed no significant variation in abundance over the years ($F_{4, 8}$ = 0.371, p = 0.823) (Fig. 1). However, and as highlighted before, the *A. obstetricans* trend has also been mediated by the presence of *Ranavirus* in some of the sites (see Chapter IV), confounding the results of this study, which seemed not to be the case for the *S. salamandra* population. In any case, the slope observed between 2010 and 2011 can still be attributed uniquely to *Bd*, since the first outbreak of ranavirosis took place later in 2011 (Chapter IV).

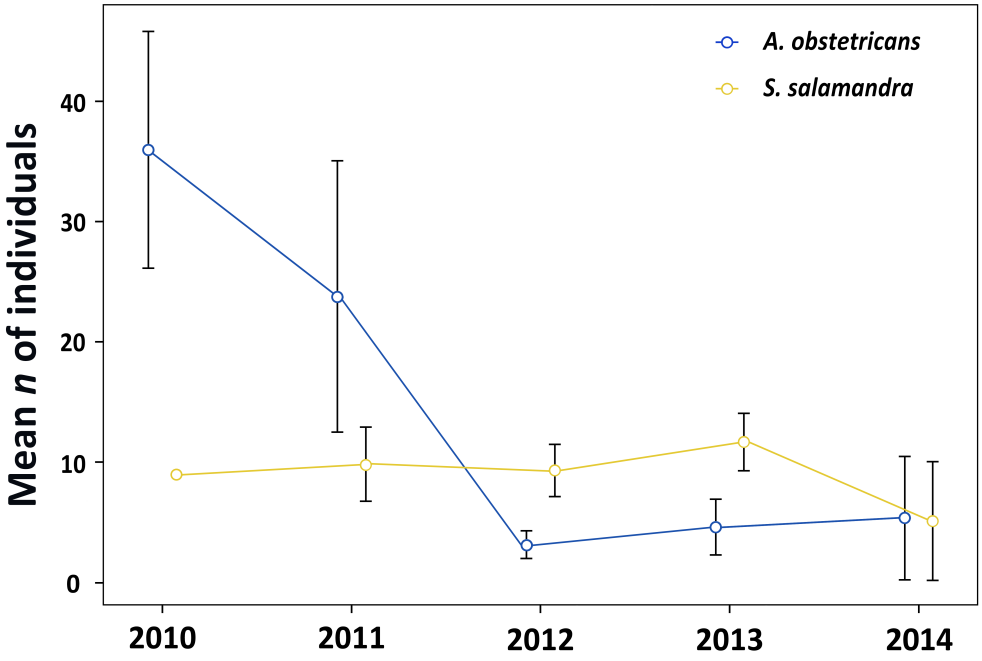

Figure 1. Abundance of two amphibian species (*Alytes obstetricans* and *Salamandra salamandra*) across five *Bd*-positive sites in Serra da Estrela (Portugal) over five years (error bars: 95% CI). On top of chytridiomicosis, *A. obstretricans* were affected by ranavirosis outbreaks (see Chapter IV).

Between 2010 and 2014, 95 individuals of fire salamander (83 larvae and 12 juveniles and adults) were sampled for *Bd*, but yielded no positives across the Natural Park. This contrasts with the scenario observed for the *A. obstetricans* population in the upper region of Serra da Estrela where *Bd* presence has been confirmed (318 larvae and 84 post-metamorphs sampled) and individuals were heavily infected (GE: 0.01-34200 (mean ± SD: 1935.9 ± 5556.0, n = 101).

3.3.2. Artificial exposures and responses

Before the experiment there were no significant differences between experimental groups for larval weight (ANOVA $ToWt^{1/3}$: $F_{3, 26} = 0.142$, $p = 0.934$) and length (ANOVA SVL: $F_{3, 27} = 1.320$, p = 0.288). After the experiment, the weight gain did not differ among experimental groups (ANCOVA: $F = 1.799$, $d.f. = 3$, $p = 0.174$), but length gain differed (ANCOVA: $F = 4.815$, $d.f. = 3$, $p = 0.009$): post hoc tests indicated differences only between the high dose treatment and all the others. Larvae exposed to a higher dose were the only treatment with overall negative length gain (Fig. 2).

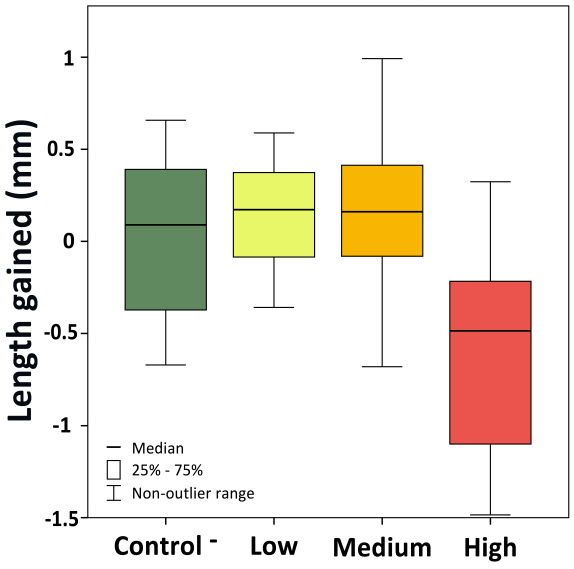

Figure 2. Box-plots displaying the length gained (mm) by individuals of *S. salamandra* larvae allocated to each experimental group that had been exposed to different loads of lab-produced *Bd* zoospores. The difference between the high dose treatment and the rest is significant at 5%.

No infections were detected in any experimental groups at the end of the experiment, although the positive control group had a *Bd* prevalence of 25% with two individuals positive for *Bd* out of 8 exposed ones. However, GE values were very low: 1.96 and 0.79 (mean ± SD: 1.37 ± 0.83). No animals died during the course of the experiment. However, on day 24 after first exposure, 50% (n = 4) of the animals in the high dose treatment started to show clinical signs such as skin sloughing, balance/ equilibrium problems (struggling to maintain a stable posture in the water column). The same signs

were also observed in the positive control group (50% of the group, *n* = 4), but not for the rest of the experimental groups.

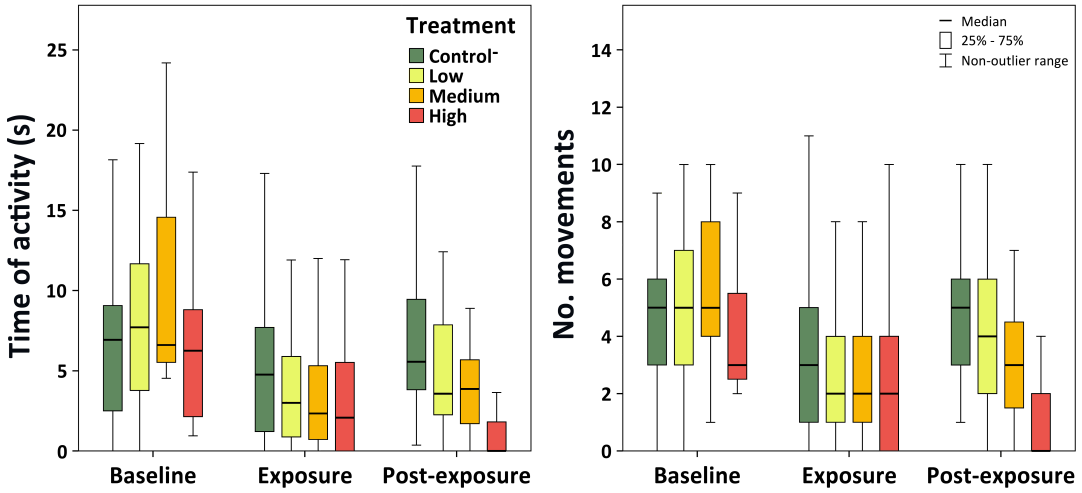

Figure 3. Distribution of *S. salamandra* larvae activity (time of activity and number of movements in 1 minute of observation) when artificially exposed to *Bd* (treatments graded by *Bd* load) at each phase of the experiment. Outliers excluded from the graph.

Exposure to *Bd* had a differential effect throughout time (the phases of the experiment) affecting both the number of movements and the time of activity, with both variables overall decreasing in a dose dependent manner (Table 1, Fig. 3). As expected, before starting the experiment (*Baseline*), there was no significant difference in the activity of individuals allocated to the different experimental groups ($p > 0.05$; Table 1). During the period of exposure to *Bd* (*Exposure*) there were marked differences in the number of movements and time of activity between experimental groups ($p < 0.01$ for both variables; Table 1). Pairwise contrasts showed that control larvae presented significantly higher number of movements and time of activity ($p < 0.05$ for both variables) when compared to all other experimental treatments. After the period of exposure to *Bd* (*Post-exposure*) differences remained evident in the number of movements and total active time between the experimental groups ($p < 0.01$; Table 1). Pairwise contrasts showed significant differences ($p < 0.05$) between all the pairs, except control larvae and the low exposure group (no. movements: $p = 0.544$; time of activity: $p = 0.086$) and low-medium (no. movements: $p = 0.111$; time of activity: $p = 0.443$). Overall, after the baseline phase controls showed a higher activity pattern throughout the experiment.

Table 1. Results of the general linear mixed models for the activity level of the *S. salamandra* larvae when artificially exposed to different *Bd* loads throughout the study and in each of the three different phases of the study.

Factor	*d.f.*	No. movements. min^{-1}		*d.f.*	Time (s) of activity. min^{-1}	
		F	*p*		*F*	*p*
treatment	3, 511	5.186	**< 0.01**	3, 515	3.022	**0.03**
phase	2, 511	11.729	**< 0.01**	2, 515	7.338	**< 0.01**
phase*treatment	6, 511	2.893	**< 0.01**	6, 515	3.128	**< 0.01**
Study phase						
Baseline	3, 511	0.346	0.792	3, 515	1.889	0.130
Exposure	3, 511	4.017	**< 0.01**	3, 515	6.673	**< 0.01**
Post-exposure	3, 511	11.797	**< 0.01**	3, 515	10.845	**< 0.01**

3.4. Discussion

Our study presents two species playing different roles in the fungal pathogen dynamics. In this system, *S. salamandra* larvae develop, grow and share the water bodies with highly *Bd*-infected *Alytes obstetricans* tadpoles (resulting in hundreds of dead toadlets) without getting infected. Conversely, we provide experimental evidence that *S. salamandra* larvae can be infected by *B. dendrobatidis*. However, the infection has only been recorded in our positive control group after multiple exposures to extremely high doses of *Bd* zoospores. These results contribute to explain what we found in natural conditions in Serra da Estrela, where salamanders seem not to mediate spillover or act as a reservoir for the disease. Yet, some inconsistency emerges when comparing *Bd*-resistance across other fire salamander populations (see below).

3.4.1. Multi-host pathogen and infectivity boundaries

Frequently, chytrids are described as host-specific parasitic fungi with only one preferred substrate (Karling, 1977; Ibelings *et al*., 2004). Nevertheless, some species are able to persist wherever susceptible substrate and/or hosts are present (Karling, 1977; Ibelings *et al*., 2004). The ability to infect hundreds of species coupled with high genetic diversity from a recent global emergent recombinant lineage (Farrer *et al*., 2011; Bataille *et al*., 2013) turned *B. dendrobatidis* into a multi-host generalist pathogen, with a wide breadth of environments in which it may persist (Fisher *et al*., 2009b), an assumption supported by evidence of inducible plasticity in pathogen growth and development (Woodhams *et al*., 2008). So why is *Bd* not taking advantage of a potential host species at Serra da Estrela,

such as the fire salamander? The situation is complex but, in this system, host range can be associated with several factors, including host and pathogen phylogeny, and life history traits (see 3.4.2) (e.g. Malpica *et al.,* 2006).

Pathogens often vary in infectivity among different host species, and tend to infect hosts that share traits i.e., phylogenetically similar to each other, which make them susceptible to the same pathogens (Freeland, 1983; Davies & Pedersen, 2008). This has been referred to as the species barrier (Blancou & Aubert, 1997). Patterns of species declines in amphibian communities infected by *Bd* suggest a taxonomic selectivity (Smith *et al*., 2009; Bancroft *et al*., 2011), with some species relatively less prone to infection and development of disease.

In cross-inoculation experiments with chlorococalean algae, Gromov *et al*. (1999) found that some chytrid fungal strains (of *Rhizophydium*) had very broad host ranges, whereas some were rather narrow. Often in phytoplankton, the attachment (and encystment) of parasitic chytrids' (Chytridiomycetes) zoospores onto a particular host or group of algae indicates that specific signals are involved in the attraction of zoospores (Powell, 1994). It is plausible to hypothesise the same for *Bd*, where host response varies with pathogen isolate (Fisher *et al*., 2009a). Differences in virulence among isolates could be attributed to evolving host specificity or environmental specialization (Rosenblum *et al*., 2008; Fisher *et al*., 2009a).

Few reports mention a possible mediation of *Bd* in salamander population declines (e.g., Rovito *et al*., 2009). As a group, caudates have been understudied with regard to *Bd* susceptibility, although newts and salamanders may be able to persist with or clear *Bd* infections to a greater extent than do anurans (Davidson *et al*., 2003; Baláž *et al*., 2014). Wild *Ambystoma tigrinum* were the first salamanders being reported to be positive to *Bd* in southern Arizona, USA (Davidson *et al*., 2003). Later on, individuals exposed to *Bd* zoospores in lab-controlled environment were still positive for chytrid 60 days following first contact. However, after a set of experiments, the team also concluded that exposure to *Bd* did not always lead to mortality and the animals appeared to recover from the infection. Similar results have been obtained with several other salamanders in North America and Europe with animals being able to clear the chytrid infection (particularly when lab setup mimics natural conditions; see e.g. Chinnadurai *et al*., 2009; Vasquez *et al*., 2009; Weinstein, 2009; Cheatsazan *et al*., 2013). Relatively more severe host responses to *Bd* were recorded in wild individuals of the endangered Sardinian newt *Euproctus platycephalus* exhibited patchy, discoloured skin and loss of digits (Bovero *et al*., 2008).

At Serra da Estrela we found great dissimilarity in host species response to parasite

natural exposure: highly infected *A. obstetricans* tadpoles and dead metamorphs (resulting in a population crash) and no records of infection in *S. salamandra*, with a fairly stable population. This can be explained by different degrees of susceptibility to the pathogenic chytrid in both species. Moreover, higher prevalence of *Bd* infection in *Alytes* tadpoles, also with comparatively higher GE values, confirms once more the high susceptibility of this species (Bosch *et al*., 2001; Rosa *et al*., 2013; Chapter II). However, even a single highly susceptible host species like the common midwife toad, may exhibit strong variation in the prevalence of infection across small geographic scales, since other factors play a role of the host-pathogen dynamics (Schmeller *et al*., 2014).

In our system, the presence of a relatively resistant host species afforded no dilution benefit to a highly susceptible species, i.e. did not have the capability to positively alter infection dynamics in that susceptible host. This contrasts with the results obtained for other systems such *Ribeiroia ondatrae* on *Bufo americanus* (as susceptible host) co-housed with *Hyla versicolor* and/ or *Rana clamitans* as decoy hosts (Johnson *et al*., 2008), but even in a *Bd* system, with *Anaxyrus boreas* (as susceptible host) co-housed with *Pseudacris regilla* and/ or *Rana cascadae* (Searle *et al*., 2011a).

3.4.2. Life history traits preventing infection

Similarly to *S. salamandra* (this study), the congeneric Alpine salamander (*Salamandra atra*) has constantly shown absence of infection by *Bd* (Lötters *et al*., 2012). However, resistance to *Bd* seems not to be a consistent trait across fire salamanders and *Bd* has actually been recorded in wild populations of *S. salamandra* in the Pyrenees, France (F. Clare pers. com.) and at Guadarrama National Park (Spain), where the populations have experienced a significant decline (Bosch & Martínez-Solano, 2006; Medina *et al*., in press). But the ability of *Bd* to infect salamanders in these places with high prevalence raises some questions: is it simply a matter of time of exposure (e.g., Carey *et al*., 2006)? Production of skin secretions (Melzer & Bishop, 2010)? Differences in microbial skin composition (Woodhams *et al*., 2007)? A combination of both? Life history traits and environmental constrains might be in the epicentre of this differences. In fact, larvae of *S. salamandra* in Guadarrama and the Pyrenees (high elevation sites) often overwinter (Bosch & Martínez-Solano, 2006), experiencing a longer larval stage and thus an extended period of contact with chytrid zoospores in water (Medina *et al*., in press). In springtime, the larvae observed in Serra da Estrela don't usually reach over 30 mm SLV (25.5 ± 2.4, n = 13), contrasting with the much larger overwintering larvae in Guadarrama (up to 43 mm SVL; 34.8 ± 4.4, n = 18) for the same time of the year, with most adults breeding in late Fall in Serra da Estrela vs.

springtime in Guadarrama. This prolonged exposure at Guadarrama or other high-elevation sites may then increase the chances of infection. This has been shown for the midwife toads in Serra da Estrela, Guadarrama and Pyrenees, where larvae often overwinter, reaching high levels of infection and developing chytridiomycosis vs. lower infectivity or absence of *Bd* in low elevation areas where tadpoles don't overwinter (Bosch *et al*., 2001, Walker *et al*., 2010; Rosa *et al*., 2013).

Salmandra salamandra can possibly be extremely efficient at blocking epidermal colonization by *Bd* even when exposed to a highly concentrated and virulent dose of zoospores, until it reaches the threshold of time of exposure. A physiological approach would help in understanding if *S. salamandra* larvae are equipped with efficient skin secretions for killing *Bd* zoospores, as observed in other species (e.g. *Speleomantes* species; see Pasmans *et al*., 2013). Genetic differences between populations (and actual different sub-species: *S. s. gallaica* in Serra da Estrela vs. *S. s. almanzoris* in Guadarrama; Steinfartz *et al*., 2000) may also play a role here and should not be discarded (Luquet *et al*., 2012).

Lastly, Serra da Estrela is an amphibian diverse region with a mixture of frogs and salamanders, raising the possibility that some might serve as a pathogen reservoir. Salamanders' abundance is relatively low in higher elevation sites (which was also shown in the 1990's by Brito-e-Abreu *et al*., 1994; see also Malkmus, 1985) and our data suggests that spillover is not occurring between this species and *Alytes*. However, infected common toads (*Bufo spinosus*) and Perez's frogs (*Pelophylax perezi*) are frequently found sharing the same habitats. This reality contrasts with the salamander population in Guadarrama, where the species is fairly common (Martínez-Solano *et al*., 2003), thus likely acting as a reservoir host (Medina *et al*., in press). Reservoir hosts play an important role in sustaining epidemics even when the density of susceptible hosts is low.

Coupled with the results shown by Baláž *et al*. (2014) our data helps in clarifying the host range boundaries of this pathogen, suggesting a taxonomic selectivity towards Alytidae, leaving *Salamandra* individuals out of infection risk, at least under Serra da Estrela environmental conditions.

3.4.3. Costs of exposure: sub-lethal effects

Pathogens can reduce host growth rates and make them more susceptible to other stress factors or causes of mortality (Dobson & Crawley 1994; Parris & Cornelius, 2004). Exposing *S. salamandra* larvae to a highly virulent *Bd* strain and in a manner that resulted in potentially lethal infection in a susceptible host resulted in persistent infection of only a low number of salamanders in the positive control group.

Warkentin *et al*. (2001) conducted the first experiments measuring the costs associated with fungal parasitism. The authors showed that pathogens can influence the timing of life history transitions, accelerating hatching of embryos (with all the consequent costs involved; see e.g., Buckley *et al*., 2005; Warkentin, 2011), allowing the survival of individuals despite infection. We detected a response that is consistent with other salamander responses to *Bd* (Venesky *et al*., 2010; Pasmans *et al*., 2013): infection with *Bd* was not associated with mortality as infection cleared in the course of time, and/or the pathogen was not even able to colonize larvae skin.

However, sub-lethal costs were found in salamander individuals that were able to resist/ clear infection, indicating that successful immune responses to infection come at a cost. For instance, SVL gain was negative in the high dose group, and was also more related with disorder and lethargy than infection status, which highlights the importance of looking at natural history traits. After 24 days of first exposure, half of the salamanders in the high dose treatment showed clinical signs such as skin sloughing, lethargy, balance and hovering problems. Even in the absence of infection, *B. dendrobatidis* releases a chemical that can cause host pathology (McMahon *et al*., 2013). Overall costs were accrued in a dose-dependent manner during the larval stage.

Exposures to *Bd* zoospores also had effects on the behaviour and activity patterns of larvae. Activity decreased significantly with the increase of zoospore dose, with individuals becoming more lethargic. This pattern is expected when looking at other stress tolerance experiments conducted with newt larvae: Griffiths *et al*. (1993) showed that under sub-lethal acid conditions feeding behaviour was suppressed in palmate newts (*Lissotriton helveticus*) and smooth newts (*L. vulgaris*), and snapping behaviour inhibited.

The effects observed in *S. salamandra* exposed to *Bd* suggest that the costs of mounting an anti-pathogen immune response and/or tissue repair mechanisms are still considerable. An individual may balance these costs via trade-offs between investment in immunity and growth. Such a trade-off is predicted (see Boots *et al.*, 1999) and has previously been identified in other larval amphibians exposed to *Bd*, such as common toads (*Bufo bufo*), where mass was the predictor (Garner *et al*., 2009, 2011). Overall, *Bd* exposure might have indirect negative impacts on populations of fire salamander through reducing individual fitness, but our study suggests that these could be ignored to a certain extent for purposes of conservation (at least in areas where the populations do not overwinter). However, other chytrids might impose serious conservation challenges to the species as it has been reported in the Netherlands where *Batrachochytrium salamandrivorans* is causing

mass die-off in *S. salamandra* populations (Martel *et al*., 2013; Spitzen-van der Sluijs *et al*., 2013).

3.4.4. Final considerations

The amphibian chytrid fungus has the ability to affect individual host growth and survival without requiring infection. Detection of *Bd* in a population and estimation of prevalence should not be assumed to be reliable indicators of risk or impact of disease for that host species. Our data suggests that for *S. salamandra* this pathogen may act as a biotic limiter, rather than a host extirpator.

Understanding patterns of host-pathogen dynamics is essential for designing management efforts to mitigate the negative effects of *Bd* and infectious agents (Muths *et al*., 2008; Murray *et al*., 2009; Rohr & Raffel, 2010). Haydon *et al*. (2002) argue that disease-control measures must be directed at the reservoir in order to increase to success of the action. The overwintering behaviour during larval stage seems to be a key trait that ensures longer exposition to the pathogen. *Pelophylax perezi* often have active larvae able to spend the winter in the water, sometimes under a thick layer of ice (García-París, 1989; Egea-Serrano *et al*., 2005). This species appears to be overinfected (Baláž *et al*., 2014; Rosa *unpublished data*) and thus may represent an overall better reservoir for *Bd* than (non-overwintering) salamander. Sampling of water frogs and/ or other species with overwintering larvae may be informative because of their widespread distribution and abundance.

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Chapter IV

Asynchronous emergence of two potentially lethal pathogens in an amphibian assemblage

Asynchronous emergence of two potentially lethal pathogens in an amphibian assemblage

Abstract. In recent years we have been witnessing an increase in reports of emerging diseases mediating declines across different taxa around the world. This may reflect greater vigilance, but anthropogenic alteration of the environment and ecosystems will likely mediate further outbreaks of emerging pathogens. Chytrids and ranaviruses have been linked to massive die-offs among the amphibians. Amphibian chytrid (*Batrachochytrium dendrobatidis*, *Bd*) was first recorded in 2009 in Serra da Estrela (Portugal) and involved in the decline of common midwife toads in the higher elevation region. Although also present in lower elevation areas, chytrid was not visibly causing mortality among the amphibian populations. Monitoring of the Serra da Estrela amphibian assemblage revealed the asynchronous emergence of a second pathogen, a *Ranavirus*. The amphibian assemblage was surveyed between 2011 and 2014 assessing the presence and distribution of *Ranavirus* in the park, as well as understanding patterns of pathogen emergence and differential mortality. Two sites with similar geo-climatic features and history of *Bd* presence were selected to test the impacts of the novel strain of *Ranavirus*, PNTRV: one with yearly outbreaks of ranaviruses, another where no outbreaks had been recorded. Skeletochronology was used to detail any age or life stage specific patterns in pathogen preference. Furthermore, sequencing data allowed inferring evolutionary relationships among Serra da Estrela ranavirus and various other ranaviruses described for Iberia, as well as representatives from the different clades. Our study shows amphibian host declines in multiple species revealing the impact of the emerging *Ranavirus*. This strain was highly virulent and mediated a series of annual mass die-offs with no cumulative or amplified effects, despite a steady prevalence of chytrid overtime. Data recorded prior to the first outbreak, as well as a comparison with the uninfected site, revealed the collapse of the amphibian assemblage in relation to ranaviral disease emergence. The new pathogen is a member of the CMTV-like clade and, similar to other closely related Iberian *Ranavirus*, it seems to have the capacity to infect multiple hosts, leading to massive die-offs across taxa, life stages and the altitudinal range.

4.1. Introduction

Emerging infectious diseases are those that have recently increased in incidence or geographic range, affected new host populations, recently been discovered or are caused by newly-evolved pathogens (Daszak *et al*., 2000). In recent years chytrids and ranaviruses have been in the spotlight due their increasing contributions to morbidity and die-offs among amphibians around the world (Duffus & Cunningham, 2010; Chinchar & Waltzek, 2014).

The most serious impacts of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*) have been described for the Neotropical region of the Americas contributing to the disappearance of dozens of species of harlequin toads (genus *Atelopus*) as well as frogs and salamanders (La Marca *et al*., 2005; Lips *et al*., 2006). However, some authors have suggested that the majority of populations where *Bd* exists declines do not occur (Daszak *et al*., 1999), or are limited to an ecologically predisposed group of species (Daszak *et al*., 2003). This has been supported by field observations such as mountain yellow-legged frog (*Rana muscosa*) populations in Sierra Nevada (Fellers *et al*., 2001), or common midwife toads in Iberia and French Pyrenees (Walker *et al*., 2010; Rosa *et al*., 2013; Chapter II), where few species within the amphibian assemblage seem to be affected and/ or effects are limited to an altitudinal envelop.

Double-stranded DNA viruses of the family Iridoviridae, genus *Ranavirus* are emerging pathogens with broad geographical and host ranges (Schock *et al*., 2008; Chinchar *et al*., 2011). These pathogens are known to infect ectothermic vertebrates including fish, amphibians and reptiles, leading to ranaviral disease and ranavirus-related mass mortality events in all three classes (Chinchar *et al*., 2009). This capacity may facilitate the persistence of ranaviruses in the environment and its spread to other species and geographic regions (Chinchar & Waltzek, 2014), explaining the apparent increase in *Ranavirus* epizootics.

Different strains of this virus with different degrees of virulence have been described, and prevalence varies between host species, location and season (Cunningham *et al*., 2007; Hoverman *et al*., 2011; Stöhr *et al*., 2015). Nonetheless, *Ranavirus* infections are often associated with epidemics that may reoccur annually, but host populations appear to persist (Brunner *et al*., 2004; Greer *et al*., 2005). However, a recent study by Price *et al*. (2014) seems to contradict the general trend, describing catastrophic consequences at the level of amphibian host communities. While the host populations are unlikely to go extinct solely due to influence of the pathogen, their numbers may drop down to low

levels, making specific species much more susceptible to stochastic events (Lafferty & Gerber, 2002).

With relatively few exceptions, most of the literature on *Ranavirus*-mediated die-offs reports single events or mortality in captive populations with no analyses of host population trends and dynamics in the face of the emerging threat. In fact, several authors have identified the need for long-term monitoring studies that track local amphibian populations over time to quantify the impacts of (recurring) die-offs on recruitment and population size (e.g. Hoverman *et al*., 2012; Gray & Miller, 2013).

While monitoring an amphibian assemblage in Serra da Estrela (Portugal) after mass die-offs due to chytridiomycosis (Rosa *et al*., 2013; Chapter II), we recorded an episode of unusual mortality in a site where *Bd* was present, although not associated with signs of disease or mortality. Water quality analyses showed that parameters were within the standards (Laurentino *et al*., in press), thereby discarding the possibility of environmental contamination. Mortality accompanied by lesions was noticed across different species present in the pond, thus contrasting with the scenarios previously observed in chytrid outbreaks in Serra da Estrela, where the pathogen has been associated with mortality in higher elevation areas, affecting mainly the midwife toad (*A. obstericans*; Rosa *et al*., 2013; Chapters II & III). Clear similarities between the observed pattern of mortality and the mass die-offs in Spain (Picos de Europa and Galicia; Price *et al*., 2014) raised the possibility of a new outbreak of *Ranavirus* infection. However, the asynchronous emergence of the two lethal amphibian pathogens with associated mortality offered a unique chance to better understand their dynamics within a multi-host community.

This study provides a record of population trends in chytrid positive sites tracked over time following the asynchronous emergence of a second disease. The amphibian assemblage at Serra da Estrela was surveyed helping mapping the presence of *Ranavirus* in the park, enabling an understanding of the patterns of pathogen emergence and differential mortality. Here we infer evolutionary relationships of ranavirus identified at Serra da Estrela with those described for Iberia, as well as representatives from the different clades.

4.2. Material and Methods

4.2.1. Sites

Serra da Estrela is the highest mountain in Portuguese mainland territory, with a maximum altitude of 1993 m. It is an extension of the Iberian Sistema Central, being located in the

eastern part of north-central Portugal (Daveau, 1971; Mora *et al*., 2001), and comprises the largest protected area in Portugal: Serra da Estrela Natural Park (PNSE) (Fig. 1).

We focused our study on two mid-elevation main sites with similar geo-climatic features (Fig. 1): the outbreak resulting in a mass mortality event took place at Folgosinho, a 255 m^2 tank of spring water with a constant flow (Table S1). For comparison, we used a similar habitat roughly at the same elevation where mass mortality events had never been recorded: a 50 m^2 tank in the Sazes area (Table S1), which is also a spring water pond. Both tanks are approximately 1.2-1.7 m deep. Other areas of the natural park were also surveyed throughout 2011-2014 and prevalence and mortality recorded (Fig. 1; Table S1).

It is interesting to note that despite chytrid fungus being present at both sites (Folgosinh and Sazes), at least since 2010, no signs of mass mortality associated with chytridiomycosis were recorded in either caudates (*L. boscai*, *Triturus marmoratus* and *Salamandra salamandra*) or midwife toad populations (Rosa *et al*., 2013).

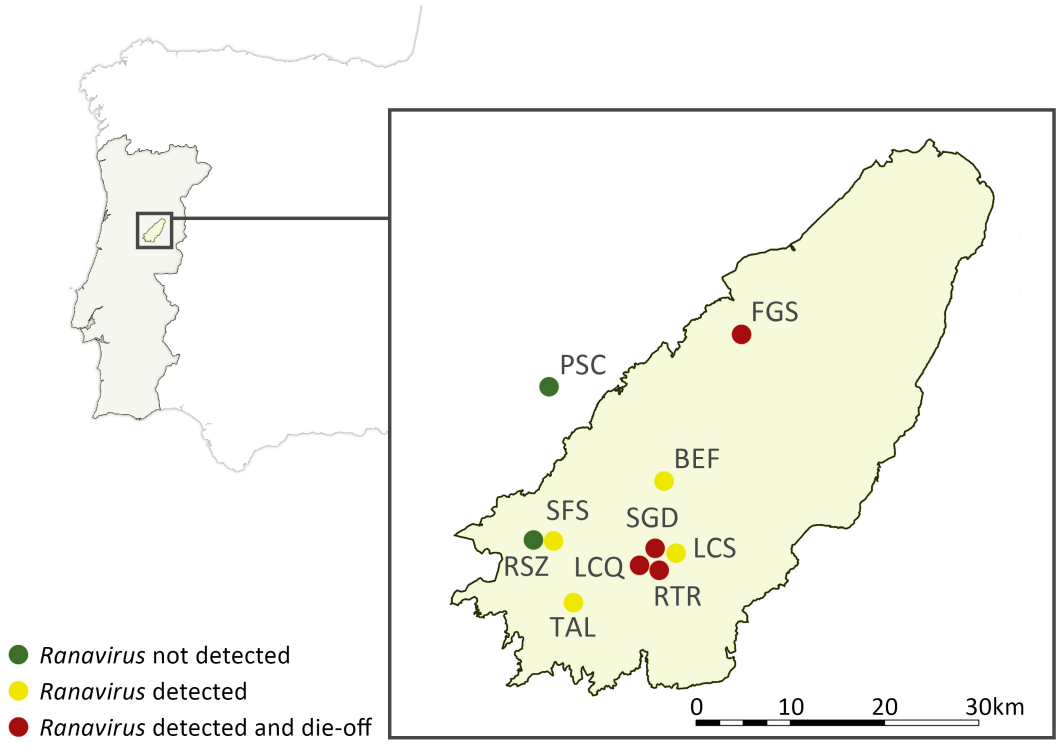

Figure 1. *Ranavirus* infection and die-offs among the amphibian assemblages in Serra da Estrela Natural Park between 2011-2014. Abbreviations key: FGS, Tanque de Folgosinho; SFS, Tanque dos serviços florestais de Sazes; RSZ, Repreza de Sazes; SGD, Salgadeiras; LCS, Lagoa dos Cântaros; RTR, Represa da Torre; LCQ, Lagoa do Covão das Quelhas; TAL, Tanque do Alvoco; BEF, Barragem da Erva da Fome; PSC, Pedreira de Santa Comba de Seia.

4.2.2. Survey

Amphibians were sampled at both sites – Folgosinho and Sazes – four times (seasons) per year for four years, from 2011 to 2014. Each sampling season consisted of 2 to 3 days of sampling (capture effort: 2 persons/ 2 hours/ day/ site), maximum 3 meters from the margins, with all stages of all species sampled by the netting. The remaining sites were sampled opportunistically to map the distribution of *Ranavirus* and detect additional mortality episodes. Sampling was performed with 50 cm diameter dip nets. Newt specimens were sexed and measured from the tip of the snout to the posterior margin of the cloaca (snout-vent length: SVL) to the nearest 0.5 mm. A piece of liver was collected from the dead specimens and stored in 70% ethanol for molecular screening of *Ranavirus* status and skin tissue for *Bd* screening (see below on 4.2.4). The right humerus and a phalanx of toe 4 of the right hind-limb were removed for skeletochronology purposes and also stored in 70% ethanol. Although the exact count of LAG is more difficult in phalanges than in humeri, it is possible to age newts through the analysis of the phalanges (Caetano & Castanet, 1993). Thus, for ethical reasons, humeri were used just to assess age of dead specimens, while a phalanx of toe 4 (right hind-limb) was collected from live specimens. This meant that no live animals had to be sacrificed and minimized any possible increase in susceptibility to infections or predation.

Visible signs of disease (skin ulceration; see e.g. Greer *et al*., 2005; Price *et al*., 2014) were noted and used to confirm status of infection in the populations. Additionally, all live specimens were swabbed for *Bd* screening and a small piece of tail tissue was clipped and stored in 70% ethanol for *Ranavirus* screening (St-Amour & Lesbarrères, 2007). Before release, an antiseptic and pain relieving solution (Bactine®, Bayer, USA) was applied to the clipped digit to reduce the chance of subsequent infection (Martin & Hong, 1991).

To prevent spreading of pathogens across sites, disposable vinyl gloves were used to handle animals. Other field equipment used during the surveys (including hiking boots) was exclusively used in the study area and was periodically immersed in a 1% solution of Virkon® (Antec International ltd., Sudbury, Suffolk, UK) before and after being used according to the protocol suggested by Speare *et al*. (2004).

4.2.3. Newt skeletochronology

The individual ages of Bosca's newts from both populations were estimated by skeletochronology, following the protocol used by Rebelo & Caetano (1995). This technique allowed detection of any age or life stage specific patterns in mortality. Humeri and

phalanges were decalcified in 3% nitric acid for 10 min (phalanx) and 50 min (humerus), cross-sectioned (14 µm width) and stained with Ehrlich's haematoxylin for 20 min (more details in Castanet & Smirina, 1990; Rebelo & Caetano, 1995). The sections were obtained after mounting on Sakura Tissue-Tek® O.C.T Compound, on a Clinicut 60 cryostat. The bone sections were fixed in a microscope slide with and posteriorly photographed and analysed.

Lines of arrested growth (LAGs) present in the periosteal bone were considered to correspond to periods of inactivity, and the zones of bone layers between LAG correspond to the periods of activity and growth (Caetano, 1990; Caetano & Castanet, 1993). A non-periodic line of metamorphosis has never been observed for this species in Portugal (Caetano & Castanet, 1993; Caetano & Leclair, 1999). Therefore, age can be estimated by directly counting the LAGs in the periosteal bone (Castanet & Smirina, 1990). The presence of additional lines, which could have been reabsorbed by the growth of the endosteal bone and the advancing cementing resorption line, was determined by measuring the average diameter of the first year LAG in the young individuals.

4.2.4. Disease screening

DNA was extracted from tissue samples (skin and liver) using the DNEasy Tissue Kit (Qiagen, Hilden, Germany). PCR to detect *Ranavirus* was performed on the DNA samples using the MP4 and 5 primers targeting the viral MCP gene (CMTV ORF 16L; major capsid protein; AFA44920) as described by Mao *et al*. (1996).

Swabs were screened for the presence of *Bd* using quantitative real-time polymerase chain reaction (qPCR), in accordance to the protocol by Boyle *et al*. (2004).

4.2.5. *Ranavirus* sequencing and phylogenetics

To understand and infer evolutionary relationships among Serra da Estrela ranavirus and various ranaviruses described for Iberia, as well as representatives from the different clades, we used DNA sequence data. Positive samples were subjected to an additional PCR reaction to amplify partial sequences from CMTV ORFs 22L (GenBank accession number AFA44926), 58L (AFA44964), 59R (AFA44965), 81L (AFA44987), 82L (AFA44988), and a region covering a noncoding sequence and the start of 13R (AFA44917). Products were visualised on a 1% agarose gel. Samples were then submitted to Beckman Coulter Genomics for Sanger sequencing of both strands (from forward and reverse primers) of DNA product.

Sequence electropherograms were viewed in CodonCode Aligner (http://www.codoncode.com/aligner/) to check the quality of base calls. Forward

sequences were reverse complemented using a bespoke unix script and aligned to reverse sequences using PRANK v.100802 (Löytynoja & Goldman, 2008). The aligned forward and reverse sequences for each sample were then viewed in Jalview 2.8 (Waterhouse *et al*., 2009) and ambiguous base calls corrected with reference to the electropherograms of both sequences. Reverse sequences were retained for subsequent analyses. Sequences were then aligned to published *Ranavirus* sequences downloaded from the NCBI nucleotide database, again using PRANK and default settings. The final alignment was visualised in Jalview and manually edited to remove gaps. The tree was constructed with MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2001) using the GTR model of nucleotide substitution. Rate variation among sites was modelled by a discrete gamma distribution with four categories. Default settings were used for Markov chain Monte Carlo (MCMC) analysis in MrBayes (750,000 generations, 4 chains, 2 runs, sample frequency = 500, and a 25% burn-in).

Additional sequences included are *Andaran Alytes obstetricans virus* (AAOV, GenBank accession number KJ703123), *Tortoise ranavirus 1* (ToRV1, KP266743), *Frog virus 3* (FV3, AY548484), *Rana grylio virus* (RGV, JQ654586), *Soft-shelled turtle iridovirus* (STIV, NC012637), *Tiger frog virus* (TFV, AF389451), *Common midwife toad virus* (Netherlands) (CMTV (nl), KP056312), *Bosca's newt virus* (BNV, KJ703122), *Common midwife toad virus* (CMTV, JQ231222), *Andrias davidianus ranavirus* (ADRV, KC865735), *Testudo hermanni ranavirus* (CH8/96, KP266741), *Epizootic hematopoietic necrosis virus* (EHNV, FJ433873), *European sheatfish virus* (ESV, JQ724856) and *Ambystoma tigrinum virus* (ATV, AY150217). Node support values are annotated on the best maximum-likelihood tree and were calculated using maximum-likelihood.

4.2.6. Statistical analysis

Density was calculated using the highest number of individuals captured in a single day per life stage per sampling season and dividing it by the area of the aquatic habitat (highest *n* / area). Mis-assignment of sex of few specimens in the field was corrected during exploratory data analysis.

We selected *L. boscai* and *A. obstetricans* (the two most abundant species) to assess any variation in the prevalence of *Bd* overtime per each site using Pearson Chi-square, and within sites using Analysis of variance (ANOVA) (IBM SPSS Statistics v.22), so we could relate any change to the emergence of the new pathogen. Prevalence of *Bd*-infected animals was calculated by dividing the number of positive individuals by the corresponding number of individuals that we tested.

Time series of counts were analysed for overall trends in population size using Poisson regression (log-linear models; McCullagh & Nelder, 1989) with the software TRIM3.0 (van Strien *et al*., 2004). We used the linear trend model with all years as change points (except for years with no observations). For each species we present an overall trend estimate (with 95% confidence intervals) calculated as the slope of the regression line through the logarithms of the indices over the whole study period. The 95% confidence interval for the overall trend estimate was used to test for significant population trends for each species (= slope +/- 1.96 times the standard error of the slope) (Pannekoek & van Strien, 2001). We followed trend classification proposed by van Strien *et al*. (2001) where (e.g.) *substantial decline/ step decline* represents a decline significantly more than 5% per year (5% would mean a halving in abundance within 15 years), and *poorly known/ uncertain*, meaning no significant increase or decline in the population, but not certain if trends are less than 5% per year.

4.3. Results

We confirmed the presence of *Bd* in both ponds with no significant variation in prevalence overtime for *L. boscai* (Folgosinho: Pearson X^2 = 0.884, *d.f.* = 3, *p* = 0.829; Sazes: Pearson X^2 = 3.357, *d.f.* = 3, *p* = 0.340) and *A. obstetricans* (Folgosinho: Pearson X^2 = 4.075, *d.f.* = 3, *p* = 0.253; Sazes: Pearson X^2 = 4.718, *d.f.* = 3, *p* = 0.194) (Fig. 2). When adding available data from 2010 (Rosa *et al*., 2013) for *A. obtetricans*, the same pattern was observed (Folgosinho: Pearson X^2 = 7.251; *d.f.* = 4; *p* = 0.123). The prevalence was also not different between the two sites for both species: *L. boscai* ANOVA: *F* = 0.086, *d.f.* = 1, *p* = 0.780; *A.*

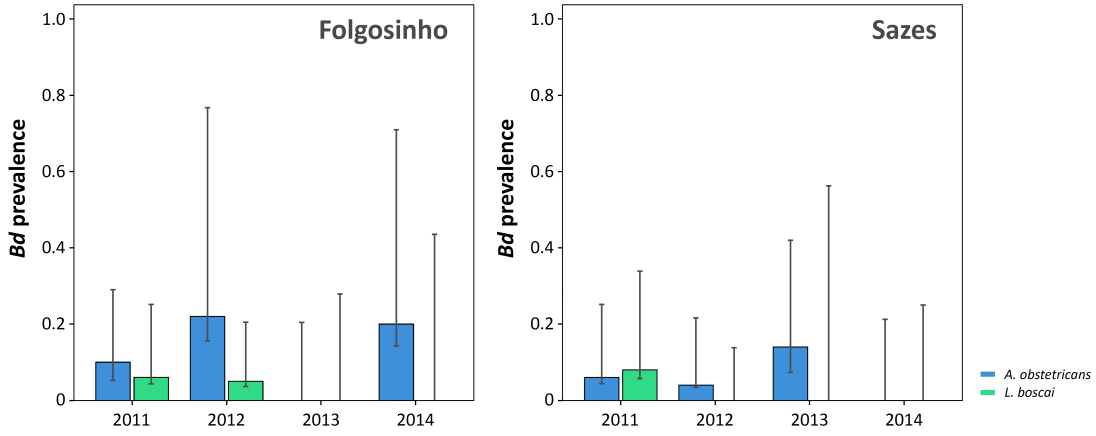

Figure 2. Prevalence of the fungus *Batrachochytridium dendrobatidis* in common midwife toad (*Alytes obstetricans*) and Bosca's newts (*Lissotrition boscai*) in two sites in Serra da Estrela (Portugal). Prevalence includes 95% confidence intervals (CIs).

obstetricans ANOVA: $F = 1.427$, *d.f.* = 1, $p = 0.277$.

The overall prevalence of *Bd* on the dead *L. boscai* found in Folgosinho across years was 5.7% (3/53), but when considering the whole sampled population of Bosca's newt (both live and dead individuals) the prevalence was 4.4% (4/92). The prevalence of *Bd* in overwintering larvae of *Alytes* never exceeded 33.3% (maximum prevalence obtained in the spring of 2014, although the sample size was very small: $n = 6$). No *S. salamandra* tested positive for *Bd* (Chapter III) and only two *T. marmoratus* were found positive. Similar prevalence results were obtained in Sazes for all of the species with *Alytes* highest prevalence not going beyond the 23% ($n = 13$) in autumn 2013.

Ranavirus is relatively wide spread through Serra da Estrena Natural Park (Fig. 1). The pathogen was first detected in the summer (August) of 2011 at the site of Folgosinho (in two live adult *Triturus marmoratus*) and the pond of Torre (several species; see table S1) by molecular diagnostics. Back then, mortality was observed in recently metamorphosed individuals of *Alytes obstetricans* and *Bufo spinosus*. Prior to the summer of 2011, all tested individuals came back negative through molecular diagnosis and no clinical signs of ranavirosis were ever observed. *Ranavirus* infection associated with disease and mass mortality was later recorded in autumn (November) of the same year, when 92.3% of the Bosca's newts found in the water were dead.

In Folgosinho, the same pattern was then repeated annually at about the same

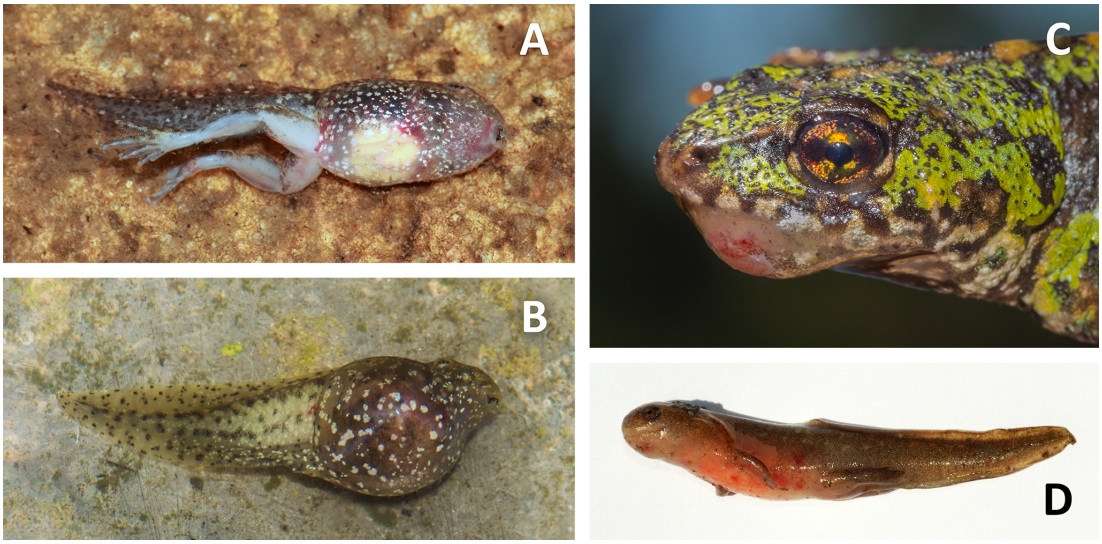

Figure 3. Ranavirosis affecting several species of amphibians in Serra da Estrela (Portugal): **A-B.** dead larvae of *Alytes obstetricans* showing systemic haemorrhaging (internal hemorrhages) and bloating; **C.** living adult of *Triturus marmoratus* with superficial and ulcerating skin lesions; **D.** *Lissotriton boscai* larva with skin haemorrhages and ulcerations.

time of the year (late summer / early autumn) during our field surveys. Numerous dead and dying adult and larval caudates (*L. boscai*, *T. marmoratus* and *S. salamandra*), and anuran amphibians (*A. obstetricans* and *B. spinosus*) were encountered and tested positive for *Ranavirus* (90% across all species, which becomes higher if focusing just on *L. boscai*: 96%). Sick/ moribund and dead animals exhibited skin haemorrhages on their ventral body surfaces, ulcerations and, in a few cases, limb necrosis, all gross signs, typical of lethal ranavirosis (Fig. 3). Together with molecular diagnostics we confirmed that infection with *Ranavirus* was associated with disease at Folgosinho (Table 1; Table S1). Infected individuals were detected in all four seasons during the time of the study.

Table 1. Epidemiology and demographic trends of two amphibian assemblages at Serra da Estrela (Portugal) after ranavirosis outbreaks. Trend classification follows TRIM v.3.53 (see also van Strien *et al*. 2001) where the multiplicative overall slope is converted into a category. The category depends on the slope as well as its 95% confidence interval.

site	**epidemiology**		**population monitoring**			
	ranavirosis infection	mortality	species	life stage	slope (SE)	population trend
Folgosinho	yes (all species)	yes	*A. obstetricans*	overwintering larvae	-0.3012 (0.0816)	Substantial decline (p<0.01) **
			L. boscai	adults	-0.9883 (0.1388)	Substantial decline (p<0.01) **
			T. marmoratus	adults	-0.5562 (0.2296)	Substantial decline (p<0.01) **
			S. salamandra	larvae	0.0375 (0.4007)	Poorly known
Sazes	yes (only on *S. salamanra* and *L. boscai*)	no	*A. obstetricans*	overwintering larvae	0.2045 (0.1174)	Poorly known
			L. boscai	adults	-0.0094 (0.0800)	Poorly known
			T. marmoratus	adults	0.1590 (0.0956)	Poorly known
			S. salamandra	larvae	-0.0373 (0.1376)	Poorly known

At the comparative site of Sazes, we recorded only four *Ranavirus*-infected individuals during the whole time of the study (out of 233 sampled; see table S1): only two were dead (both *S. salamandra* adults) and the other two (*L. boscai* adults) showed no signs of disease. In any case, no outbreak of ranavirosis was ever recorded at Sazes.

Additionally, mortality associated with ranavirosis was recorded in other places in Serra da Estrela (represa da Torre, Salgadeiras, and Covão das Quelhas: Fig. 1) where *Alytes* individuals of all life history stages (from populations recovering from a chytridiomycosis outbreak) were found dead or exhibiting signs of clinical ranavirosis. Similar to Sazes, individuals from other sites were tested positive for *Ranavirus* through molecular detection, but no individuals were found dead or showing signs of disease. These sites included a tank in Alvoco, the pond of Cântaros and Erva da Fome (Fig. 1). Individuals of other anuran species were also found positive for *Ranavirus* in those areas (*Pelophylax perezi*), but again no mortality was observed.

The skeletochronological analysis of the *L. boscai* populations showed that the same number of LAG in humeri and phalanges were confirmed for almost all the individuals where both bones were analysed; however in eight individuals (out of 210) the phalanges exhibited one LAG less, which is expected due to a natural higher rate of endosteal resorption in phalanges (Caetano, 1990). Similar results have been previously shown also for *L. boscai* (Díaz-Paniagua & Mateo, 1999). Larvae and recently metamorphosed individuals that were caught before the first season of low activity showed no LAGs. The results from this analysis showed that mortality occurred across all life stages and ages within stages making use of the aquatic environment at Folgosinho, from larval forms to recent metamorphs or sexually mature adults (Fig. 4).

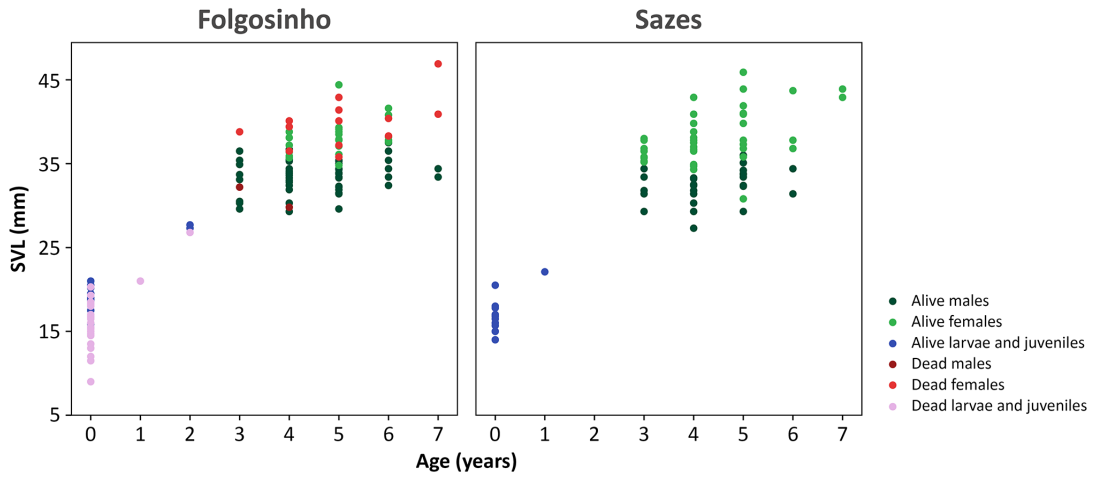

Figure 4. The relationships between age and size (snout-vent length, SVL) of *L. boscai* plotted by population, between 2011 and 2014: Folgosinho illustrates the population of newts where yearly outbreaks of ranaviruses have occurred, affecting all ages (reddish tones highlight individuals found dead and positive for the pathogen), while Sazes is used to illustrate a comparative population where outbreaks have not been recorded. Mortality in Folgosinho was found on both males and females.

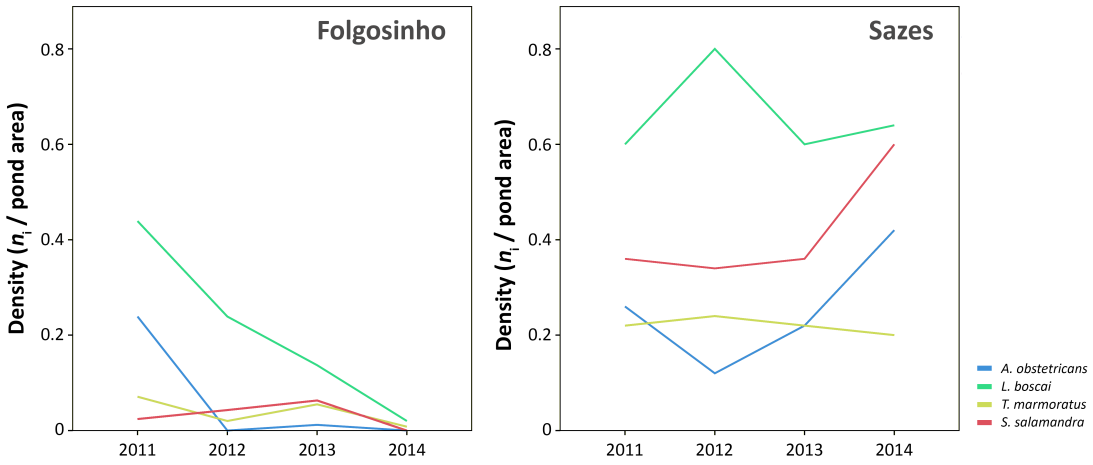

Figure 5. Population trends in two amphibian communities in Serra da Estrela (Portugal) over four years (spring counts). Folgosinho shows the density per species before (2011) and after yearly outbreaks of ranaviruses, while Sazes shows the density fluctuations of an assemblage where outbreaks have not been recorded. Life history stage targeted varied with the species but remained consistent for each site across yearly surveys: *Alytes obstetricans* (overwintering larvae), *Lissotrition boscai* (adults), *Triturus marmoratus* (adults) and *Salamandra salamandra* (larvae).

When analysing the overall trends in abundance we found a highly significant effect of time for all the species with all experiencing a sharp decline at Folgosinho (Fig. 5; Table 1). The adult population of *L. boscai* suffered a decline of 45.5% between 2011 and 2012 and 68.8% between 2011 and 2013 (Fig. 5). Unfortunately, in spring 2014 the pond of Folgosinho was cleaned by the forestry services one month before our survey, causing a dramatic disturbance in the populations, and thus compromising the interpretation of data on the population trend of the amphibian community. Thus, the results referring to that year should be interpreted carefully. The abundance of Bosca's newts in spring 2014 corresponds to a 95.5% reduction, when compared to 2011, before the *Ranavirus* outbreak (Fig. 5). The same happened to the *T. marmoratus* population, declining 72.2% on the year after the outbreak, and to *A. obstetricans*, with overwintering larvae not even being recorded in the spring of 2012 and 2014 (Fig. 5). However, this was not the case for the *S. salamandra* population, which showed no significant variation in density over the years, despite the detection of infection in a few individuals since the first outbreak (Table S1: overall prevalence in larvae since 2011 of 5.3%) (Fig. 5; see also Chapter III). In comparison, at Sazes, the trends of all the species showed no significant increase or decline (Fig. 5; Table

Figure 6. Mass mortality episode in Serra da Estrela, Portugal (summer 2013). The figure shows dead individuals of *Alytes obstetricans* and *Bufo spinosus* testing positive for both *Bd* and *Ranavirus* through molecular technique.

1), but the low number of sampling points over time and natural fluctuation of the populations did not allow a higher level of certainty to ensure stability either.

Additionally, in die-offs recorded in high elevation sites (e.g. Torre and Salgadeiras) animals often testing positive for both pathogens through molecular techniques, confounding the determination of the cause of death (Fig. 6). Midwife toads (normally dying from chytridiomycosis after metamorphoses; Rosa *et al*., 2013), were then found in the water at different developmental stages, from larvae to recent metamorphs, showing signs of infection by both pathogens (metamorphs displaying skin ulcerations and a simple reddening or sloughing of skin; Fig. 6). Additional co-infections were detected on on *Hyla molleri*, *B. spinosus* and *L. boscai*.

The sequences obtained from the viruses found in different specimens (*L. boscai*, *T. marmoratus* and *A. obstetricans*) were 100% identical to each other on all sequenced genes, and the virus was recently named *Portuguese newt and toad ranavirus* (PNTRV), a CMTV-like *Ranavirus* (Stöhr *et al*., 2015). Phylogenetic analyses places the PNTRV as a member of the "CMTV-like" viruses, closely related to the *Bosca's newt virus* (BNV) from Spain (Fig. 7).

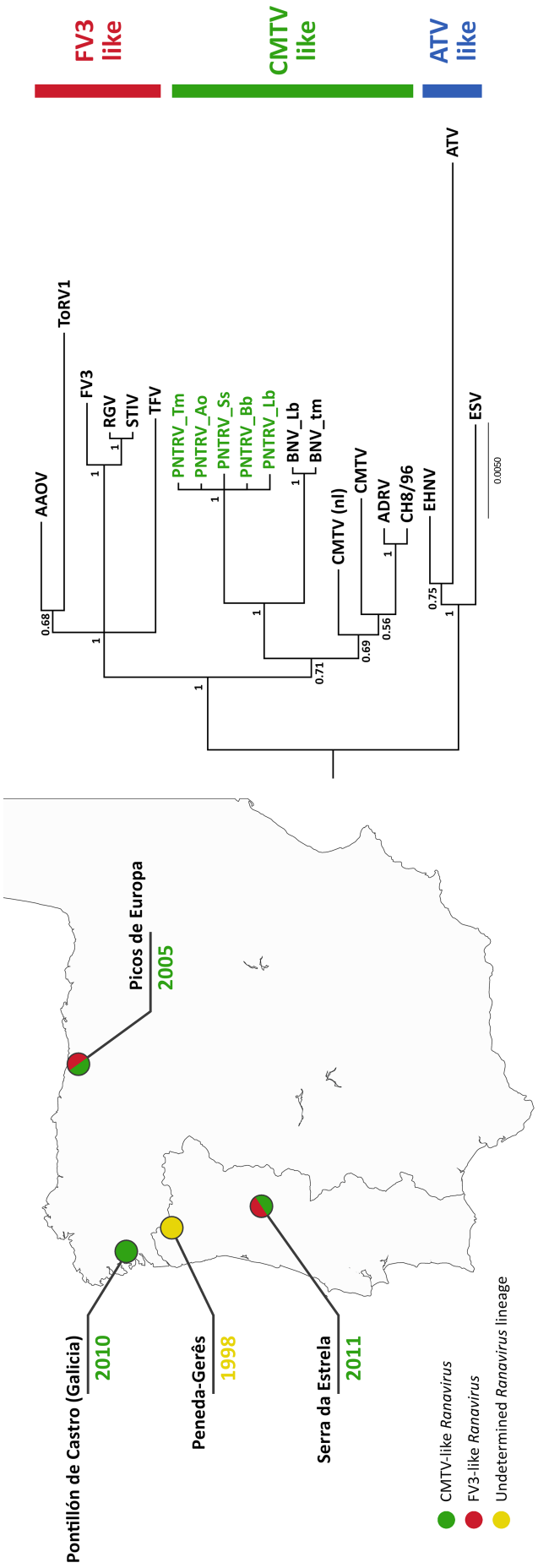

Figure 7. Phylogeography in the Iberian Peninsula inferring the evolutionary relationships between PNTRV and the known Iberian ranaviruses described for wild herpetofauna populations. The map shows first time recorded CMTV-like *Ranavirus* outbreaks leading to die-offs in amphibian communities in the Iberian Peninsula (green), with additional distribution of FV3-like *Ranavirus* (red) and one undetermined lineage (yellow). The PNTRV found in the amphibians is placed in the CMTV-like clade. Due to insufficient data, FV3-like *Ranavirus* previously described from Serra da Estrela (Alves de Matos *et al.*, 2011) was not included in this tree. Node support values are annotated on the best maximum-likelihood tree and were calculated using maximum- likelihood (bootstraps) under a GTR model of molecular evolution. Scale of branch lengths is in nucleotide substitutions per site.

4.4. Discussion

Our study provides evidence of an asynchronous emergence of two amphibian pathogens involved in multi-host mass mortality and population decline attributable to the second. A highly virulent *Ranavirus* mediated the collapse of the amphibian community. Data recorded prior to the first outbreak, as well as the existence of a comparative site with similar geo-climatic features, allowed us to understand the population trends of several hosts during the outbreaks.

Multi-pathogen systems have been understudied due to the complexity of the dynamics, but also because such events seem not to be so frequent. The available studies are greatly focused on humans (e.g. Gallay *et al*., 2006; Dallman *et al*., 2014) or agricultural systems, and so of cultural, commercial, and/ or medical importance (e.g., Grube *et al*., 2011). In any case, research being conducted largely lacks population monitoring overtime, making it harder to understand pathogens' impacts or even the properties of the interactions with the hosts' communities. Although research has being limited to few studies, amphibian chytrid and ranaviruses are known to occur and share hosts in amphibian assemblages with so far no cumulative or amplified effects reported (Schock *et al*., 2010; Whitfield *et al*., 2013).

Our system showed a new scenario where an altitudinal envelope seemed to prevent *Bd* from causing any visible damage to a mid elevation host assemblage (Rosa *et al*., 2013). However, when this multi-host community came into contact with a later emerging hyper-virulent *Ranavirus*, it experienced a general collapse. A constant (lower) prevalence of *Bd* on both populations across time reinforces the thesis of *Ranavirus* as the driver of the observed declines. On the other hand, levels of mortality increased in higher elevation sites, where all life stages were being affected (e.g. *A. obstetricans* larvae and metamorphs). Thus, field data may suggest an amplification of the pathogenicity of both parasites, an aspect that deserves more investigation.

Viruses, like some fungi and bacterial infections, can be highly lethal to naive hosts leading to mass die-offs and even local extinctions (Lesbarrères *et al*., 2011; Fisher *et al*., 2012). Although many ranaviruses demonstrate host preference (e.g., ATV and tiger salamanders; Schock *et al*., 2008), CMTV-like ranaviruses seem to be in the process of emerging in amphibian assemblages with the capacity to infect and cause significant disease and death in multiple host species (Price *et al*., 2014). This was also observed in our system where the pathogen was able to infect and cause mortality in all of the amphibian species and age classes examined, pushing the densities to alarmingly low numbers. The

field reports match the phylogenetic relationship of the virus (PNTRV) found in Serra da Estrela, considered part of the hyper virulent CMTV-like clade, where is also included in close proximity the BNV and original CMTV isolated and described from mass die-off events in Spain (Balseiro *et al*., 2010; Price *et al*., 2014). In addition to their high virulence and transmission through several routes (Hoverman *et al*., 2011; Lesbarrères *et al*., 2011), the ability of ranaviruses to exploit several biotic reservoirs with different susceptibility within the same assemblage, may allow these pathogens to remain in the system independent of population crashes. It is interesting to notice the trend observed in the *S. salamandra* population where no declines were detected (Chapter III), and that therefore may be a silent host for this virus. This may reflect the ability of this pathogen to persist in some hosts as persistent/ inapparent infections (Morales *et al*., 2010). To some degree, this mirrors the results and trends reported by Price *et al*. (2014) in northern Spain, where invasion by a CMTV-like *Ranavirus* has led to the collapse of all species.

Greer *et al*. (2008) suggested that the extinction of tiger salamanders as a result of virulent ATV *Ranavirus* is unlikely, with larval salamander populations decreasing and then recovering after ATV-driven epidemics. However, and as our data show, even if this *Ranavirus* does not drive the host to complete extinction, it can severely reduce population size to the point at which the species became highly vulnerable to stochastic events (de Castro & Bolker, 2005): one month after the pond of Folgosinho was cleaned in spring 2014, we only found five adult Bosca's newts (in the breeding season; vs. 228 in 2011) and no overwintered midwife toad larvae (vs. 126 in 2011).

Although a general sharp population decline trend has been observed in all CMTV-like *Ranavirus* outbreaks in Iberia in recent years, host heterogeneity may play an important role in the disease dynamics. While in North America there are records of ATV *Ranavirus* affecting single species of ambystomid (e.g. Jancovich *et al*., 1997; Brunner *et al*., 2004), or FV3 *Ranavirus* in the UK causing decline in *Rana temporaria* (Teacher *et al*., 2010), in Iberia we observe entire amphibian assemblages crashing (e.g. Price *et al*., 2014; this study). These emerging events are taking place in aquatic communities that include multiple species from different ectothermic vertebrate classes. Brenes *et al*. (2014) showed experimentally that reptiles and fish might serve as reservoirs for ranaviruses given their ability to live with subclinical infections. In fact, non-lethal infections have been previously documented in the Iberian rock lizard *Iberolacerta monticola* in Serra da Estrela (Alves de Matos *et al*., 2011). Although the virus strain (LMRV) detected in those lizards was genetically distinct from the virus found in this study, the role of these lizards in ranavirus

(PNTRV) persistence and emergence of new strains is unclear. Additionally, LMRV or any other FV3-like *Ranavirus* was yet undetected across the amphibian taxa in Serra da Estrela.

Transmission can happen between species, even between different classes (Bandín & Dopazo 2011), likely facilitating persistence of the pathogen in the environment. Emerging hyper-virulent *Ranavirus* strains (e.g., CMTV-like) might in this way take advantage of naïve hosts easing spill-over and species jumps - for example, predation of marbled newts upon Bosca's newts have been reported in our system (Baptista *et al*., 2015). This poses an additional threat to all lower vertebrates associated with aquatic habitats, including endemic freshwater fish only found in specific sites in Iberia (Oliveira *et al*., 2007).

Our results illustrate the second outbreak of a *Ranavirus* in Portugal causing massive die-offs in an amphibian assemblage. Froufe *et al*. (1999) reported several dead marbled newts exhibiting signs identical to clinical ranavirosis at the shores of a pond at Peneda-Gerês National Park (Fig. 7), a protected area in northern Portugal in 1998. In the following years other amphibian species present in that system were also found dead and diseased (Soares *et al*., 2003), revealing the presence of iridovirus-like particles in the underlying cells (Alves de Matos *et al*., 2008). Despite phylogenetic data not being available for this ranavirus, we can still notice a geographic pattern that mirrors the close relationship of the two CTMT-like ranavirus from western Iberia (PNTRV and BNV) forming a clade.

The progressive decline of amphibian populations in pristine and protected areas, such as Guadarrana (Bosch *et al*., 2001), Picos de Europa (Price *et al*., 2014), or Serra da Estrela (Rosa *et al*., 2013; this study) has raised concerns about the possibility of host population extirpation due to the hypervirulent nature of some pathogens. When a host population starts to decline, the pathogen's transmission rate is also expected to drop, unless there is frequency-dependent transmission, and/ or environmental reservoirs and alternative hosts (de Castro & Bolker 2005; Greer *et al*., 2008), which seems to be the most likely scenario in these multi host amphibian communities. Thus, the co-action of these two pathogens might exacerbate the effects observed if environmental and ecological conditions allow. If there are any mechanisms promoting a shift in the host-pathogen relationship favouring host survival, they still remain unsolved. Long term monitoring is thus essential to understand natural fluctuation and detect abnormal declines, as well as understand the specific needs of each pathogen or multi-pathogen system, giving time to implement an efficient mitigation and reduce the disease(s) impact.

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Supplemental Information

Table S1. Summary of samples screened for *Ranavirus* infection in Serra da Estrela, Portugal by site and year (2011-2014). Prevalence includes 95% confidence intervals (CIs). Life stages: L, larvae; M, metamorphs; J, juveniles; A, adults. Species: Ao, *Alytes obstetricans*; Bs, *Bufo spinosus*; Pc, *Pelobates cultripes*; Pp, *Pelophylax perezi*; Ri, *Rana iberica*; Hm, *Hyla molleri*; Ss, *Salamandra salamandra*; Tm, *Triturus marmoratus*; Lb, *Lissotriton boscai*.

Site	GPS	Year	Host species	Life stage	Prevalence	95% CI (range)
Represa da Torre	40°19'34.61"N, 7°36'32.07"W; 1955 m a.s.l.	2011	Ao	M	4/4 (1)	0.510-1.000
			Bs	M	9/9 (1)	0.701-1.000
		2012	Ao	M	0/1 (0)	0.000-0.794
			Bs	M	4/24 (0.17)	0.067-0.359
			Pp	M	0/2 (0)	0.000-0.658
		2013	Ao	L	16/22 (0.73)	0.518-0.868
				M	1/5 (0.2)	0.036-0.624
			Bs	M	1/1 (1)	0.207-1.000
			Pp	L	0/5 (0)	0.000-0.435
				A	0/4 (0)	0.000-0.490
Charco da Pedreira de Santa Comba de Seia	40°27'30.22"N, 7°42'36.41"W; 475 m a.s.l.	2013	Pp	L	0/3 (0)	0.000-0.561
				A	0/4 (0)	0.000-0.490
			Pc	L	0/2 (0)	0.000-0.658
Barragem da Erva da Fome	40°23'28.87"N, 7°36'1.00"W; 1450 m a.s.l.	2014	Ri	L	0/5 (0)	0.000-0.435
			Ss	L	1/1 (1)	0.207-1.000
Lagoa do Covão das Quelhas	40°19'38.55"N, 7°37'31.81"W; 1820 m a.s.l.	2013	Ao	M	0/2 (0)	0.000-0.658
			Pp	L	0/8 (0)	0.000-0.324
				J	0/2 (0)	0.000-0.658
			Ss	L	1/1 (1)	0.207-1.000
				J	0/2 (0)	0.000-0.658
			Tm	A	0/1 (0)	0.000-0.794
			Lb	M	0/1 (0)	0.000-0.794
				A	2/2 (1)	0.342-1.000
Lagoa dos Cântaros	40°20'9.43"N, 7°35'33.20"W; 1646 m a.s.l.	2012	Ao	L	0/1 (0)	0.000-0.794
			Pp	L	1/1 (1)	0.207-1.000
			Tm	L	0/3 (0)	0.000-0.561
		2013	Pp	L	0/15 (0)	0.000-0.204
				J	0/4 (0)	0.000-0.490
				A	0/3 (0)	0.000-0.561
			Tm	L	0/6 (0)	0.000-0.390
			Lb	L	1/1 (1)	0.207-1.000
Represa de Sazes	40°20'39.14"N, 7°43'21.78"W; 780 m a.s.l.	2012	Lb	A	0/1 (0)	0.000-0.794
		2013	Ao	L	0/17 (0)	0.000-0.184
			Ri	L	0/5 (0)	0.000-0.435

				A	0/3 (0)	0.000-0.561
			Ss	L	0/6 (0)	0.000-0.390
				M	0/1 (0)	0.000-0.794
			Lb	A	0/1 (0)	0.000-0.794
Salgadeiras	40°20'18.84"N, 7°36'51.59"W; 1845 m a.s.l.	2014	Ao	A	1/14 (0.07)	0.013-0.315
			Hm	A	1/1 (1)	0.207-1.000
			Pp	A	1/12 (0.08)	0.015-0.354
			Ss	A	0/1 (0)	0.000-0.794
Tanque do Alvoco	40°17'59.37"N, 7°41'21.32"W; 861 m a.s.l.	2012	Lb	A	0/1 (0)	0.000-0.794
		2013	Lb	A	1/1 (1)	0.207-1.000
Tanque de Folgosinho	40°29'37.09"N, 7°31'47.61"W; 1079 m a.s.l.	2011	Ao	L	2/2 (1)	0.342-1.000
				M	1/1 (1)	0.207-1.000
				A	0/2 (0)	0.000-0.658
			Tm	A	2/2 (1)	0.342-1.000
			Lb	L	20/20 (1)	0.839-1.000
				M	10/10 (1)	0.723-1.000
				A	40/80 (0.50)	0.393-0.607
			Ss	L	0/6 (0)	0.000-0.390
		2012	Ao	L	2/3 (0.67)	0.208-0.939
				A	3/3 (1)	0.439-1.000
			Tm	L	1/1 (1)	0.207-1.000
				A	1/3 (0.33)	0.061-0.792
			Lb	L	17/18 (0.94)	0.742-0.990
				M	4/15 (0.27)	0.109-0.520
				A	26/104 (0.25)	0.177-0.341
			Ss	L	0/10 (0)	0.000-0.278
				A	0/1 (0)	0.000-0.794
		2013	Ao	L	4/9 (0.44)	0.189-0.733
				M	0/1 (0)	0.000-0.794
				A	0/5 (0)	0.000-0.435
			Tm	L	0/1 (0)	0.000-0.794
				A	1/18 (0.06)	0.010-0.258
			Lb	L	5/9 (0.56)	0.267-0.811
				M	3/7 (0.43)	0.158-0.750
				A	10/76 (0.13)	0.073-0.226
			Ss	L	2/19 (0.11)	0.029-0.314
				M	0/2 (0)	0.000-0.658
		2014	Ao	L	0/6 (0)	0.000-0.390
				A	0/6 (0)	0.000-0.390
			Tm	A	0/2 (0)	0.000-0.658
			Lb	A	0/5 (0)	0.000-0.435
Tanque dos Serviços Florestais de Sazes	40°20'39.70"N, 7°42'52.63"W; 985 m a.s.l.	2011	Ao	L	0/4 (0)	0.000-0.490
			Lb	L	0/12 (0)	0.000-0.243
				A	0/25 (0)	0.000-0.133

	2012	Lb	L	0/36 (0)	0.000-0.096
			A	0/45 (0)	0.000-0.079
		Tm	L	0/2 (0)	0.000-0.658
			A	0/6 (0)	0.000-0.390
		Ss	L	0/3 (0)	0.000-0.561
			A	2/5 (0.40)	0.118-0.769
	2013	Ao	L	0/35 (0)	0.000-0.099
		Ri	A	0/1 (0)	0.000-0.794
		Lb	L	0/21 (0)	0.000-0.155
			M	0/2 (0)	0.000-0.658
			A	0/80 (0)	0.000-0.046
		Tm	L	0/13 (0)	0.000-0.228
			A	0/23 (0)	0.000-0.143
		Ss	L	0/34 (0)	0.000-0.102
			A	0/1 (0)	0.000-0.794
	2014	Ao	L	0/12 (0)	0.000-0.243
		Lb	A	2/12 (0.17)	0.047-0.448
		Tm	A	0/10 (0)	0.000-0.278
		Ss	L	0/3 (0)	0.000-0.561

Chapter V

How may host sex-biased mortality mediate the impact of a virulent pathogen?

How may host sex-biased mortality mediate the impact of a virulent pathogen?

Abstract. Pathogen impacts can be strongly influenced by environmental components. The periodic appearance of life-cycle events (phenology) change host availability to the pathogen in a repeatable pattern, with differences in abundance across life history stages and sexes. The effects of these cycles on the impacts of pathogens have been largely disregarded in wildlife epidemiological studies. A recent series of outbreaks of ranavirosis led to great declines of Boscas' newt populations at Serra da Estrela Natural Park (Portugal). The peculiar phenology of the studied population, with a high number of females not leaving the water after the breeding season, turn it into a suitable model to test how sex-biased mortality can affect host population persistence in the context of infectious diseases.

We explored how differences in host phenology can affect host persistence in the context of infectious diseases. Particularly we investigated 1) how the phenology of Bosca's newt (i.e. biased number of females occurring in water) mediated the impact of *Ranavirus*; and 2) we evaluated the risk of extinction of the population under different scenarios of sex-biased mortality using a population viability analysis (PVA).

Two Bosca's newt populations (one with yearly outbreaks of ranaviruses, and a comparative site where outbreaks have not been recorded) were tracked for trends over time following emergence of ranaviral disease, allowing us to assess the differential impact on both sexes. Field data showed sex-specific mortality by a hyper-virulent *Ranavirus* leading to a sharp decline of the newt population. The impact of the reported disease outbreaks was mediated by the host phenology that seems to amplify the lethal effect by causing a strongly imbalanced sex ratio among the sexually mature population. Our model projections suggest that female-biased mortality seems more likely to impact during an eventual recovery of the population after potential ceasing of mass mortality events.

5.1. Introduction

Pathogens can have differential effects on a species or population (e.g., Filotas & Hajek, 2004; Walker *et al*., 2010). These effects are generally mediated by different biotic and abiotic factors, from environmental components (Dowell, 2001; Roberts & Wiedmann, 2003; Schmeller *et al*., 2014) to host community structure (e.g., host diversity; Begon, 2008), which have the potential to alter the pathogens' prevalence and virulence. Yet, even under identical conditions, individuals or species may differ in susceptibility to the same pathogen (Searle *et al*., 2011). These differences in host susceptibility also influence the impact of the disease, and drive ecological phenomena such as pathogen amplification or dilution (Schmidt & Ostfeld 2001; Keesing *et al*., 2006).

It is likely that multiple drivers interact in complex ways in natural systems leading to seasonal patterns in hosts and pathogens, with outbreaks occurring at the same time each year (Altizer *et al*., 2006; Grassly & Fraser, 2006; Brunner *et al*., 2015). It has been suggested that certain life-history strategies may increase rates of pathogen transmission or growth (Searle *et al*., 2011). Indeed, phenology, which refers to the periodic appearance of life-cycle events (see Visser *et al*., 2010), might play a role in host-pathogen dynamics. When pathogen seasonality coincides with a particularly vulnerable phase of the host life cycle, severe consequences may arise for the host population. However, there are few empirical studies in this area, particularly those using behavioural data from natural populations.

Most epidemiological studies embracing host phenology have been conducted in plants (Dodd *et al*., 2008; Desprez-Loustau *et al*., 2010), with just a few on animal hosts. Most of the latter focus on humans: measles and influenza are probably the best-studied cases (Anderson & May, 1991). Among the few wildlife examples, Filotas & Hajek (2004) showed that the pathogen maximizes infection and dispersal at optimal abiotic conditions when its host is active. However, translating pathogen seasonality onto host population dynamics is poorly understood with emerging infectious pathogens.

European newts of the genus *Lissotriton* metamorphose from aquatic larva to terrestrial juvenile, and develop into an adult stage that alternates between aquatic and terrestrial phases (Caetano & Leclair, 1999; Brockes & Kumar, 2005). These newts show a very marked phenology, with males, females and larvae using the aquatic environment at different times, which makes them an ideal model to address this host-pathogen interaction perspective. If a pathogen was able to cause high mortality in all life history stages at all times, an extinction would occur almost immediately. However, different sexes

and stages may be exposed to pathogens at different times, thus allowing for a better persistence of the host population.

Ranavirus is an emerging pathogen with a broad geographical range (Schock *et al*., 2008; Chinchar *et al*., 2011), recently increasing in incidence and affecting new host populations (Gray *et al*., 2009). This pathogen is known to infect fish but also amphibians and reptiles, contributing to mass mortality events in all three vertebrate classes (Chinchar *et al*., 2009; Chinchar & Waltzek, 2014). However, virulence and prevalence varies with host species, location and time of the year (Cunningham *et al*., 2007; Hoverman *et al*., 2011; Stöhr *et al*., 2015).

Ranavirus epizootics have often been reported to be annual, but host populations seem to persist (Brunner *et al*., 2004; Greer *et al*., 2005). Yet, many studies lack long-term monitoring data and hence a thorough assessment of the pathogen impacts. In the Great Smoky Mountains National Park, repeated die-offs involving multiple amphibian species have been occurring for over 10 years, but population-level effects are unknown (Sutton *et al*., in press). On the other hand, recently emerged CMTV-like strains seem to be contradicting that general trend in Iberia, leading to catastrophic consequences at the community level (Price *et al*., 2014; Chapter IV). Hyper virulent recurring episodes of disease mortality are expected to decrease the expected time to extinction, although it is unlikely to be the sole driver of this event. Pathogen action may thus cause host populations to drop down to low levels, becoming more susceptible to stochastic events (Lafferty & Gerber, 2002).

A recent series of yearly outbreaks of ranavirosis led to massive impacts on the amphibian assemblages in Serra da Estrela Natural Park (Portugal) (Chapter IV). The authors revealed the impact of a novel strain of this generalist *Ranavirus* pathogen (*Portuguese newt and toad ranavirus* (PNTRV), a CMTV-like *Ranavirus*; Stöhr *et al*., 2015) that has the capacity to infect multiple different hosts, affecting the entire amphibian assemblages, including the newt species in freshwater habitats.

Bosca's newt population trends were tracked over time following emergence of ranaviral disease and allowed us to assess the differential impact on both sexes. Thus, making use of this host-pathogen system, here we explore how differences in phenology can affect host persistence in the context of infectious diseases. More specifically, we investigate 1) how the phenology of a host species (Bosca's newt) mediates the impact of a generalist pathogen that causes sex-specific patterns of mortality; and 2) evaluated the risk of extinction of a population under different scenarios of sex-biased mortality mediated by a virulent pathogen.

5.2. Material and Methods

5.2.1. Sites

Serra da Estrela is the highest mountain in Portugal's mainland territory, with a maximum altitude of 1993 m. Part of the Iberian Sistema Central, it is located in the eastern part of north-central Portugal (Daveau, 1971; Mora *et al*., 2001), and comprises the largest protected area in Portugal: Serra da Estrela Natural Park (PNSE).

Disease outbreaks resulting in mass mortality events started to take place in the area of Folgosinho, in a 255 m^2 tank of spring water with constant flow (40°29'37.09"N, 7°31'47.61"W, 1079 m a.s.l.) in the early autumn of 2011 (Chapter IV). As a comparable site we used another spring water tank at the same elevation where *Ranavirus* outbreaks have never been recorded: the 50 m^2 tank in the Sazes area (40°20'39.70"N, 7°42'52.63"W; 985 m a.s.l.). Both tanks are approximately 1.2-1.7 m deep, located in mountain slopes with the same orientation (facing west), and roughly 23 km apart.

Chytrid fungus (*Batrachochytrium dendrobatidis*), a fungal pathogen commonly associated with amphibian die-offs in Iberia and elsewhere, has been present in both sites at least since 2010, but no signs of mass mortality associated with chytridiomycosis were have been recorded before (Rosa *et al*., 2013) or during the study (Chapter IV).

5.2.2. Host phenology

Bosca's newt, *Lissotriton boscai* (Lataste, 1879) is endemic to the western half of the Iberian Peninsula and relatively common in suitable habitats (Pleguezuelos *et al*., 2002; Loureiro *et al*., 2008). This species is subject to increasing anthropogenic threats, including loss of breeding pools (by drainage for agriculture and urbanization), introduction of non-native predators, and also local outbreaks of emerging pathogens (Soares *et al*., 2003; Arntzen *et al*., 2009).

The migration of *L. boscai* into or out of the ponds occurs on an individual basis; that is, there are with no mass movements (Caetano & Leclair, 1999). In Serra da Estrela, at around 1000 m elevation males start migrating to the ponds in November, although most wait until February. Mating takes place in April-June and larvae are present between May-December (although a few might overwinter). The adults start leaving the ponds in June, but some individuals (mostly females) stay in the water year-round (Fig. 1). Phenology is similar to the population living at 1000 m analysed by Caetano & Leclair (1999) in another mountain system in Northern Portugal. For instance, the males migrate to the ponds earlier than females, also leaving first and if the conditions are favourable some individuals may

spend most of the year in water (Fig. 1). In Serra de Sintra, Malkmus (1980-81) also recorded aquatic activity throughout the year in the *L. boscai*, and the percentage of males in the water would drop down to 10% between August and December.

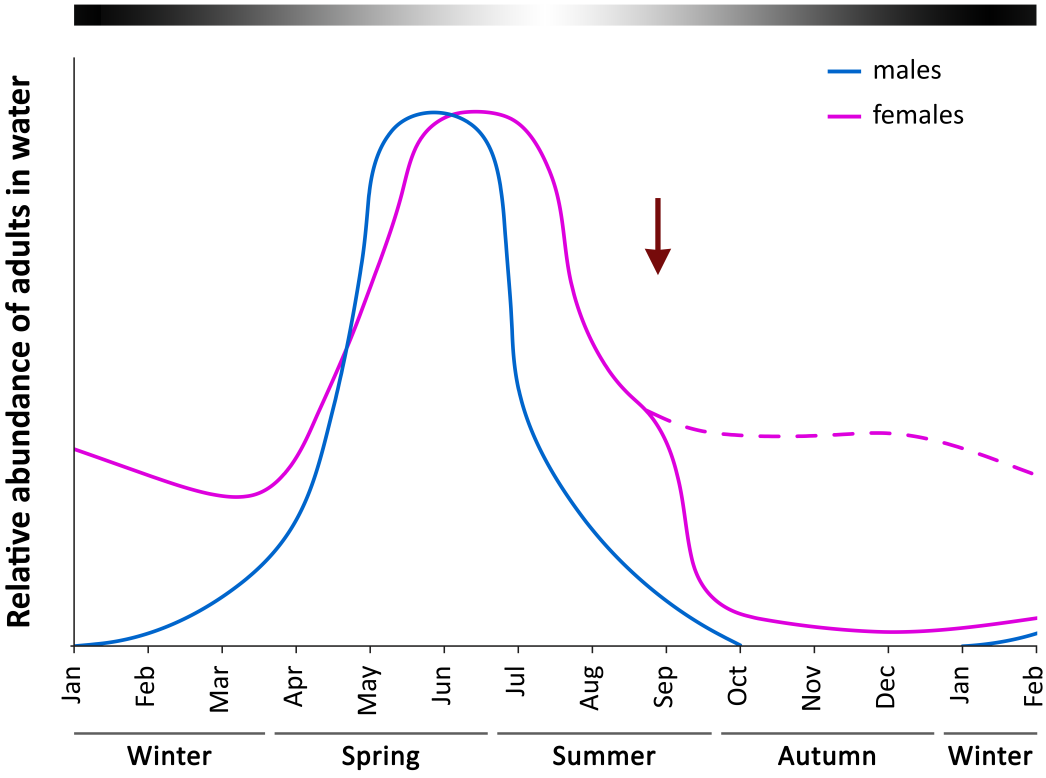

Figure 1. Schematic phenology of the host species, Bosca's newt (*Lissotriton boscai*) in Serra da Estrela (Portugal) in ponds located at about 800-1100 m of elevation showing relative proportion of males and females in the water throughout the year. Arrow indicates time of the first outbreak of ranavirosis (2011) and dashed line illustrates the expected relative abundance of females in the absence of outbreak (based on relative data from previous years and other similar ponds, collected as part of ongoing amphibian monitoring studies; Rosa *et al.*, 2013; Chapter III). Smooth curves were fitted using Autodesk AutoCAD 2016 and edited on Adode PhotoShop CS6. Top bar represents the temperature throughout the year from cool (dark) to warm (light).

5.2.3. Survey

Surveys of these two selected sites enabled us to better understand the differential impact of *Ranavirus* over time, both in terms of demography and sex ratio. Newts were thus sampled four times (seasons) per year for 4 years, from 2011 to 2015 (but with missing data between summer 2014 and winter 2015). Each sampling season comprised 2 to 3 days of

sampling (capture effort: 4 persons/ hour/ site). The highest count per site (within the same season) was considered the peak abundance and later used in the analyses. Sampling was performed with dip nets of approximately 50 cm diameter. To assess the disease status of each living individual, a small piece of tail tissue was clipped, and from dead specimens a piece of liver was collected instead. All tissue samples were stored in 70% ethanol for *Ranavirus* assay (St-Amour & Lesbarrères, 2007). Before release, antiseptic and pain-relieving solution (Bactine®, Bayer, USA) was applied to the clipped tail to reduce the chance of subsequent infection (Martin & Hong, 1991).

To prevent the spread of pathogens across sites, disposable vinyl gloves were used to handle animals. Other field equipment used during the surveys (including hiking boots) was exclusively used for the study area and was periodically immersed in a 1% solution of Virkon® (Antec International ltd., Sudbury, Suffolk, UK) before and after use according to the protocol suggested by Speare *et al*. (2004).

5.2.4. Disease screening and sequencing

DNA was extracted from tissue samples using the DNEasy Tissue Kit (Qiagen, Hilden, Germany). PCR to detect *Ranavirus* was performed on the DNA samples using the MP4 and 5 primers as described by Mao *et al*. (1996). To characterize the virus, the positive samples were sequenced using the viral MCP (major capsid protein) gene (Mao *et al*., 1996) and blasted in GenBank. Amplification products were submitted for Sanger sequencing, and sequences were archived in GenBank.

5.2.5. Population Viability Analyses

A population viability analyses (PVA) were used to evaluate the risk of extinction and population recovery under different scenarios of *Ranavirus* outbreaks. Models were built using VORTEX v. 10.0, which is primarily used to model the probability of extinction of small populations and the relative effects of differing actions and/or perturbations, allowing for individual age- and sex-structured demographic models (http://www. vortex10.org; Lacy *et al*., 2005).

Demographic parameters for the PVA were estimated as follows:

Population Structure. As the newt population includes both terrestrial and aquatic stages, the term *population* was used here to refer to a unit that encompasses a breeding pond and its immediate terrestrial hinterland. Bosca's newts become sexually mature at the age of 3 years (Caetano & Leclair, 1999; Diaz-Paniagua & Mateo, 1999; Chapter IV).

However, some sub-adult animals (i.e., non-breeding newts) may also return to the water each year.

We ran our models as population-based, incorporating environmental stochasticity, and assuming that environmental variation in reproduction and survival were correlated. However, no inbreeding depression was assumed, since it has been suggested that inbreeding has no major role in the extinctions of amphibian populations (Sjögren, 1991; Halverson *et al*., 2006).

Spatial Structure and Dispersal. Dispersal habits of *L. boscai* are poorly known, yet a close relative, *L. italicus*, has limited dispersal capabilities (Ragghiami & Wake, 1986). On the other hand, and similar to other species (e.g., *L. helveticus*; Montori & Herrero, 2004), the young terrestrial newts might show some capacity to disperse. As the aquatic habitat of Folgosinho is isolated, and in the absence of more indicative data, we assumed no dispersal (dispersal rate of 0 %) and thus models were based on a single population.

Reproductive system. As male newts may mate with several females during the course of a breeding season (Verrell, 1989), a polygynous mating system was incorporated. However, the balanced sex ratio and the frequent occurrence of male interference (Faria, 1995) may limit male capacity to mate with multiple females (see Verrell (1989) for additional physiological constraints).

Reproduction and mortality rates. Males and females had identical age structures and survival rates (Chapter IV). Published estimates of fecundity for *L. boscai* range from 100 to 250 eggs per female (see Barbadillo *et al*., 1999; Galán & Fernández, 1993; Salvador & García-París, 2001; Brea *et al*., 2007). Assuming a 1:1 operational sex ratio, and considering 25% infertility and mortality (Orizaola & Braña, 2004), we obtain a mean number of larvae per female of 131.25 (SD = 56.25). Overall, 8.9% survivorship was expected during larval development (conservative estimation based on data available from Orizaola & Braña (2003) and several other studies on newts: Bell & Lawton, 1975; Kusano, 1981; Miaud, 1993). Some predation certainly occurs in our populations (e.g. Rosa *et al*., 2012; Baptista *et al*., 2015) justifying the high mortality in these early life stages.

Since no specific adult annual survival data were available for *L. boscai*, this parameter was estimated from our data based on age composition (obtained from skeletochronology; see Chapter IV) using the Robson & Chapman (1961) method. Additionally, other studies on closely related species and other newts sharing similar habitats were also taken into account, particularly for the sub-adults annual survival (Bell, 1977; Hagström, 1979; Miaud *et al*., 2000; Griffiths & Williams, 2001; Jakob *et al*., 2003).

Knowing that Bosca's newts breed for the first time at 2 or 3 years and assuming that the individuals found in the tank are representative of the breeding adult population (similarly to great crested newts: Griffiths & Williams, 2001), we estimated an annual adult mortality of around 0.30, and sub-adult mortality at 0.39. Standard deviation of 0.1 was added to the survival estimates of adults and 0.28 of sub-adults (SD obtained after: Bell, 1977; Griffiths & Williams, 2001; Jakob *et al*., 2003) since the annual survival of juveniles is more variable than adult survival (Bell, 1977; Griffiths & Williams, 2001).

In other species of European newts it is possible that a proportion of females does not breed every year (e.g., Griffiths, 1984; Accordi *et al*., 1990; Díaz-Paniagua, 1998). For the populations in Doñana (Spain), Díaz-Paniagua & Mateo (1999) have also suggested that females have a lower reproductive rate than males. Thus, for modelling purposes, we assumed that 80% of the adult females reproduce, with 20% SD due to environmental variation (conservative data also based on Orizaola & Braña, 2003).

Initial population size. In spring 2011 we used capture-mark-recapture (CMR) to assess absolute population density at Folgosinho site (Rosa, unpubl. data). Because of the low numbers of recaptures a *Fisher-Ford* method of population *estimate* was used (Fisher & Ford, 1947). We obtained an estimated initial population of about 2650 individuals (using a stable age distribution), considering this number our starting population size (N_o) in the models.

Density-dependence. Density-dependent effects are still poorly understood in newts. However, some studies have shown that populations are normally regulated in a density-dependent way (e.g., Hagström, 1979). Gill (1978, 1979) demonstrated that adults (particularly females) of red-spotted newt (*Notophthalmus viridescens*) had the highest annual survival in uncrowded ponds. A simple ceiling model was then set to affect all vital rates and considered to be based on the abundance of all stages. The carrying capacity (K) was set at 100% higher than the initial population size (i.e., $K = 2\ N_0$), based on data estimated from the comparative population from Sazes.

Catastrophes. There are a number of factors that can result in catastrophic mortality of these newts, such as fungal infections of eggs and predator introductions (e.g., Cruz *et al*., 2006). Water tanks are subject to emptying for cleaning, maintenance or firefighting, which may severely reduce adult and larval survival. Even if the pond does not dry completely, desiccation will lead to a reduction or loss of recruits as a result of increased predation or intraspecific competition in a crowded habitat. These events seem to occur about once every 3 years (personal obs.). This was modelled assuming a

desiccation risk of 33.33%. In years when this event takes place the recruitment of 1-year-old newts was reduced by 90% (estimation based on our field data coupled with Griffiths, 2004).

Simulation Scenarios. VORTEX allows for the removal of individuals ("harvest"), simulating scenarios such as research-related removals, culling or mimicking hunting (Lacy *et al*., 2005). To simulate the effects of disease-induced mortality (ranavirosis) on population stability we tested different "harvest" rates (see below) for larvae and adults, while subadult stages (corresponding to the terrestrial phase and thus not accounting for population reduction) remained constant (0). Models were thus constructed considering different scenarios of sex-biased mortality. Seven scenarios were setup:

Scenario 1: no *Ranavirus* outbreaks;

Scenario 2: annual *Ranavirus* outbreaks within the first 5 years of simulation with a biased effect on females; at-risk stages were larvae and >3-year-olds; disease outbreak causes constant annual offtake of 40% of larvae and 55% of all mature individuals (90% females and 10% males);

Scenario 3: same as for *Scenario 2*, but with *Ranavirus* outbreaks affecting equally both males and females: constant annual offtake of 40% of larvae, 55% of all mature individuals (equal number of males and females);

Scenario 4: annual *Ranavirus* outbreaks within the first 10 years of simulation with a biased effect on females; at-risk stages were larvae and >3-year-olds; disease outbreak causes constant annual offtake of 40% of larvae and 55% of all mature individuals (90% females and 10% males);

Scenario 5: same as for *Scenario 4*, but with *Ranavirus* outbreaks affecting equally both males and females: constant annual offtake of 40% of larvae, 55% of all mature individuals (equal number of males and females).

Scenario 6: annual *Ranavirus* outbreaks within the total time of simulation (20 years) with a biased effect on females; at-risk stages were larvae and >3-year-olds; disease outbreak was a constant annual offtake of 40% of larvae and 55% of all mature individuals (90% females and 10% males);

Scenario 7: same as for *Scenario 6*, but with *Ranavirus* outbreaks affecting equally both males and females: constant annual offtake of 40% of larvae, 55% of all mature individuals (equal number of males and females).

Scenarios #2 and #3 were set to simulate what has been observed in our system but also in other Iberian CMTV-like *Ranavirus* systems, where the pathogen has been present in the community for at least 5 years (Price *et al*., 2014). Scenarios #4 to #7 project different scenarios reflecting mid- to long-term pathogen persistence in the population (as long as there are suitable hosts and/ or environmental conditions), a reality suggested by Teacher *et al*. (2010). The difference in terms of female-biased vs. equal sexes removal reflects what we observed in our system compared to a scenario where females and males are affected in the same way.

Stochastic simulation of demographic (and epidemiological) processes was carried out with 10000 estimates (iterations) and extinction risk was measured as the proportion of populations going extinct within 20 years. We considered extinction to have occurred when only one sex remained.

5.2.6. Statistical analysis

Density was calculated dividing the highest number of individuals captured in a single day per life stage per sampling season by the area of the aquatic habitat (highest *n* / area). Sex ratio was expressed as a proportion i.e. males/(males + females). Comparisons of sex ratios were carried out with software IBM SPSS 20.0 (IBM corp. Chicago, USA), using Generalized Linear Models (site*time as fixed effects) assuming a binomial error distribution with a logit link function. Post-hoc pairwise comparisons were performed using Bonferroni correction.

The nonparametric Kruskal-Wallis test was used to test differences of extinction probabilities between scenarios of the PVA. Post-hoc testing was then performed through Dunn-Bonferroni test to ascertain which pairs of groups differed significantly from one another.

5.3. Results

5.3.1. Ranavirosis and mortality

Ranavirus with disease and associated mass mortality was first observed in November (autumn) 2011, where 92.3% of the individuals of Bosca's newt found at Folgosinho were dead. The same scenario occurred annually at about the same time of the year (late summer / early autumn) during our field surveys (Fig. 2). Numerous dead and dying adult and larval Bosca's newts were encountered and tested positive for *Ranavirus* (96%). Sick and dead/ moribund animals exhibited all gross signs typical of lethal ranavirosis (see Chapter IV). Mortality was not recorded during springtime but some positives were

detected (5%, *n* = 20 in 2013). However, when occasional visits were made to the pond early in the year, dead individuals were observed in the water (> 50 in January and > 10 in March 2012). In contrast, no outbreak of ranavirosis or mass mortality events were ever recorded at the comparable site at Sazes (Fig. 2).

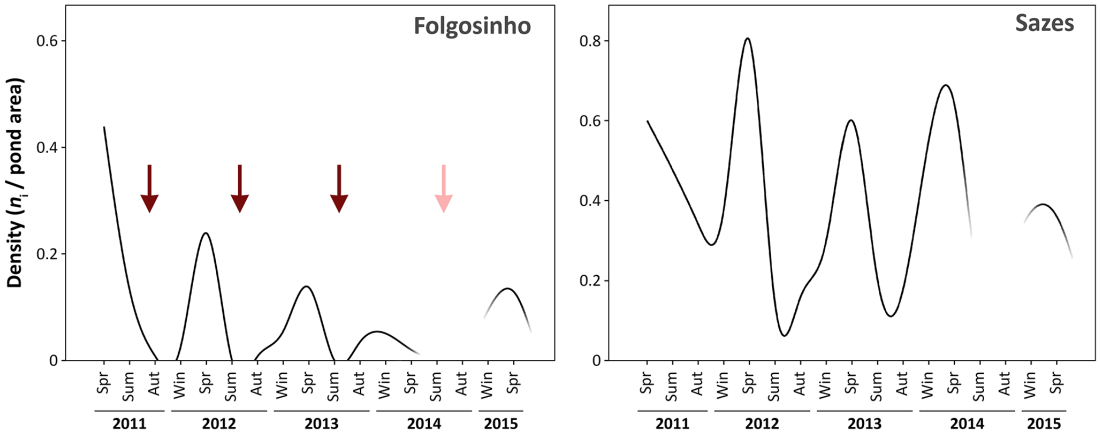

Figure 2. Density of adult Bosca's newt (*Lissotriton boscai*) in two sites in Serra da Estrela (Portugal) over five years (spline interpolation with data missing between summer 2014 and winter 2015): Folgosinho shows the density of newts with yearly outbreaks of ranaviruses (arrows indicate timing of the outbreaks), while Sazes shows a natural population fluctuation in an area where outbreaks have not been recorded.

As shown by Rosa *et al*. (Chapter IV), mortality was found across all life stages making use of the aquatic environment (Fig. 3), with the adult population suffering a decline of 45.5% between 2011 and 2012 and of 68.8% between 2011 and 2013. In spring 2014 we observed a reduction of 95.5% of the Folgosinho population when compared to 2011, before the *Ranavirus* outbreak (Fig. 2; see Chapter IV).

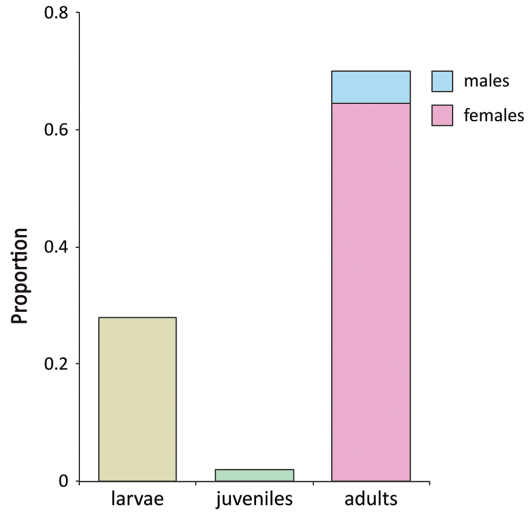

Figure 3. Proportion of individuals by life stage recovered dead in the pond of Folgosinho (Serra da Estrela, Portugal) during ranavirosis outbreaks between 2011-2015.

5.3.2. Population sex ratio

Populations of newts from both sites showed no differences in the sex ratio in springtime 2011, before the first outbreak of *Ranavirus* (Fig. 4 and Table 1). At the control site (Sazes), where no outbreaks of *Ranavirus* were detected, there was no significant change in the sex ratios of *L. boscai* (spring: Wald X^2 = 3.328; *d.f.* = 4; p = 0.505; autumn: Wald X^2 = 1.075; *d.f.* = 2; p = 0.584; Fig. 4; Table 1) over the period of this study. Later that year (2011), during the first outbreak in Folgosinho, 91.7% of the sexually mature *L. boscai* individuals found dead were females.

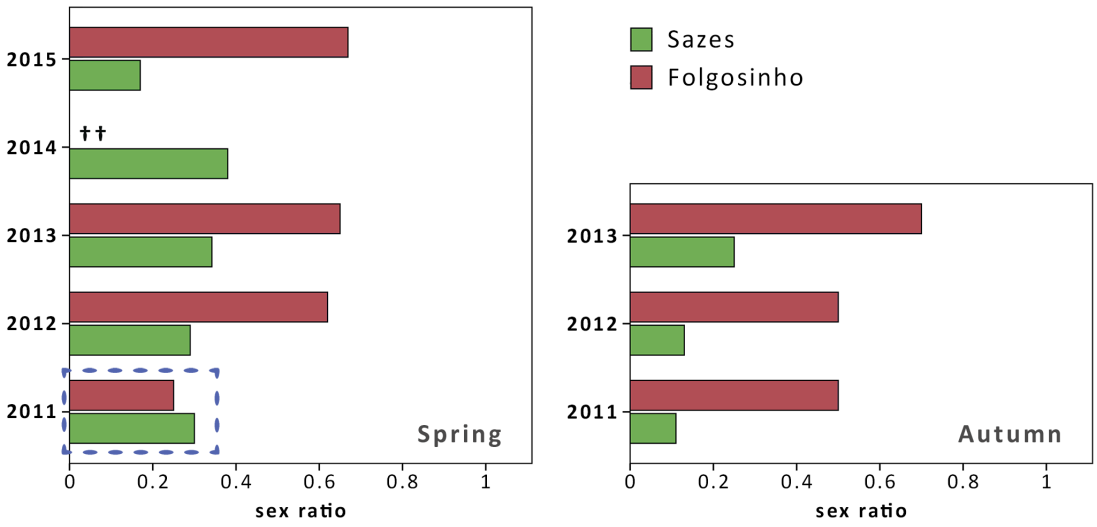

Figure 4. Comparative sex ratio of sexual mature Bosca's newt (*Lissotriton boscai*) in two sites in Serra da Estrela (Portugal) over five years. Folgosinho shows the sex ratio of a population facing yearly outbreaks of ranavirosis after spring 2011, while Sazes shows the expected sex ratio of a population where outbreaks have not been recorded. Sex ratio is expressed as the proportion of males/(males + females) in two different seasons: spring and autumn. Blue dashed rectangle highlights the sex ratio for both populations before the first outbreak of ranavirosis where differences between them were not significant (*L. boscai* $p > 0.05$). †† No data was considered for Folgosinho site in spring 2014.

In the following years we noted a drastic change in the proportion of males and females arriving in the tank. This was translated into a complete reversal of the sex ratio of the population, shifting from 25% males in late spring 2011 to over 60% in the subsequent years (Fig. 4). We thus observed a significant effect of time after the first outbreak on the sex ratio in Folgosinho in springtime (Wald X^2 = 45.209; *d.f.* = 3; $p < 0.001$), with a marked

difference between the first year and subsequent years (Table 1). The data collected in the first autumn (2011) was already influenced by the outbreak, and when looking at the proportion of male *L. boscai* found in the autumn across years we did not detect differences (Wald X^2 = 0.638; *d.f.* = 2; p = 0.727; see Table 1). Larvae comprised a smaller proportion of dead individuals over 5 years (28%) than adults (70%) (Fig. 3). In any case, Fig. 3 highlights an overall unbalanced sex ratio with 64.4% of all the dead being females.

Table 1. Pairwise comparisons of springtime sex ratios between two populations of *Lissotriton boscai* over time at Serra da Estrela (Portugal) using a Generalized Linear Model (site*year). Folgosinho: yearly outbreaks of *Ranavirus* record (2011 represent the sex ratio before the first outbreak); Sazes: no *Ranavirus* outbreak recorded. A p value < 0.05 was considered significant and highlighted in bold after Bonforroni correction.

Folgosinho	2011	2012	2013	**Sazes**	2011	2012	2013	2014
2012	**< 0.01**			2012	1.00			
2013	**< 0.01**	1.00		2013	1.00	1.00		
2014	††	††	††	2014	1.00	1.00	1.00	
2015	**< 0.01**	1.00	1.00	2015	1.00	1.00	1.00	1.00

Sites	year	Bonferroni adj. p value
Folgosinho*Sazes	2011	1.00
	2012	**< 0.01**
	2013	**0.02**
	2015	**< 0.01**

†† No data was considered for Folgosinho site in spring 2014.

5.3.3. Risk assessment and population viability

The models suggested that the *L. boscai* population has a 40% chance of persisting (i.e., probability of extinction > 0) over the course of the 20-year simulation, if it does not experience declines for more than 10 years (Table 2). However, with no female-biased mortality, this probability increases to over 85%.

Probability of extinction differs between the different scenarios (K-W: X^2 = 55.163; *d.f.* = 6; p < 0.001; Table 3). In all the projected scenarios with disease outbreaks, there is a rapid decline of the population within the first 5 years to approximately 15% of the initial population size (pre-outbreak), resembling the values observed in the field (95.5% decline until 2014 and 70.6% by 2015 compared to 2011; Fig. 2; see also Chapter IV). If the outbreaks stop after the first 5 years of disease, there is a good chance that the population

Table 2. PVA outputs from simulation of disease outbreaks in a population of *Lissotriton boscai* under seven different disease mortality offtakes. Adult (55%) and larvae mortality (40%) is constant across scenarios #2-#7 (varying in time and sex offtake) but absent in #1. PE: probability of population extinction after 20 yrs; GR: stochastic growth rate; N: mean population size after 20 yrs; TE: time to first population extinction; SD: standard deviation.

Scenario	PE	GR ± SD	N ± SD	Median TE
#1. no *Ranavirus* outbreaks	0.001	0.206 ±0.606	4104.95 ±1551.87	0
#2. female biased mortality within 5 yrs	0.132	-0.026 ±0.661	2325.63 ±2112.10	0
#3. equal sex mortality within 5 yrs	0.009	0.046 ±0.677	3390.51 ±1916.13	0
#4. female biased mortality within 10 yrs	0.580	-0.293 ±0.634	237.41 ±714.17	12
#5. equal sex mortality within 10 yrs	0.164	-0.149 ±0.687	769.65 ±1246.74	0
#6. female biased mortality within 20 yrs	0.984	-0.516 ±0.393	0.12 ±0.84	11
#7. equal sex mortality within 20 yrs	0.880	-0.452 ±0.472	0.67 ±2.44	16

grows and recovers (Fig. 5). However, a female biased mortality will make this recovery much slower, with the population reaching just 87% of the initial abundance in 20 years (scenario #2), while equal disease risk between the sexes will allow for a recovery to N_0 in less than 15 years (scenario #3; Table 3). Five years after the last outbreak (i.e. year 10), projection #3 presents a population 1.8 times larger than #2 (Fig. 5). A short period of female-biased mortality (5 years) results in an effect as strong as mid- and long-term persistence of equal mortality (Fig. 6; Table 3).

Ten years after annual outbreaks of disease, our simulations show a population decline of over 99% compared to N_0 (scenarios #4 to #7; Fig. 5). If disease happened for 10 years with higher mortality of females, by year 20 the population will recover slightly, but is still reduced to about 9% of what it was before any outbreak, and over three times smaller compared to scenario #5. Ten years of female-biased mortality are not significantly different from 20 years of disease exposure and mortality (regardless any bias) in terms of probability of population extinction overtime (Fig. 6; Table 3).

When looking at the two projections where disease never ceased, we observed no chance of recovery in the two scenarios (#6 and #7; Fig. 5). Both scenarios predict a high

likelihood of population extirpation (98% and 88% respectively) with a median time to extinction of 11 years when mortality is female biased vs. 16 years on equal sex mortality (Table 2).

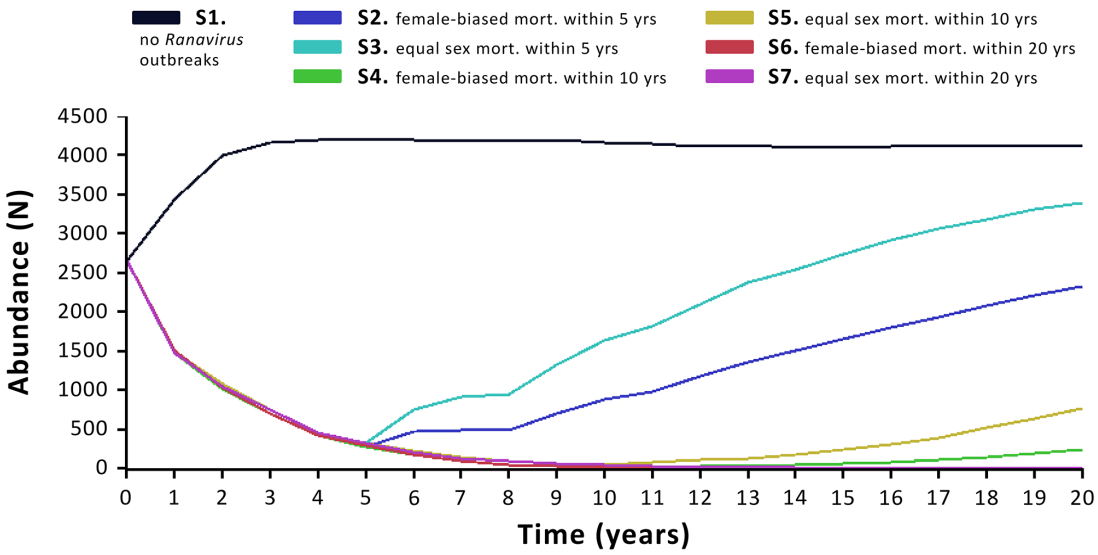

Figure 5. 20-year population projection for Bosca's newt (*Lissotriton boscai*) in VORTEX under seven different disease scenarios with no management. The figure shows a faster recovery of the affected population in scenarios of balanced mortality among sexes when compared to a female-biased mortality.

5.4. Discussion

Our study shows sex-specific mortality by a highly virulent pathogen leading to a sharp decline of the newt population. We found that the impact of the reported disease outbreaks was mediated by the host phenology that seems to amplify the lethal effect observed by causing a strongly imbalanced sex ratio among the sexually mature population.

Although recovery was observed in larval tiger salamander populations after ATV-driven epidemics (Greer *et al*., 2008), ranaviruses seem to meet the conditions required to cause host extinction (Miller *et al*., 2011). A recent study by Earl & Gray (2014), modelling the likelihood of extinction of a closed population of wood frogs (*Lithobates sylvaticus*) exposed to *Ranavirus*, showed that extinction could theoretically occur as quickly as 5 years with annual exposures. Our analyses and models suggest that even if the recurring ranavirosis outbreaks do not drive the host to complete extinction, they severely reduce the effective population size to the point at which the species becomes highly vulnerable to

Table 3. Dunn-Bonferroni test between pairs of different scenarios generated from PVA, to look at significant differences in probability of extinction. A adjusted *p* value < 0.05 was considered significant and highlighted in bold.

Scenario	1	2	3	4	5	6
2	**< 0.01**					
3	1.00	0.26				
4	**< 0.01**	1.00	**< 0.01**			
5	**0.03**	1.00	1.00	0.31		
6	**< 0.01**	1.00	**< 0.01**	1.00	0.10	
7	**< 0.01**	1.00	0.22	1.00	1.00	1.00

stochastic events (de Castro & Bolker, 2005). As an example of stochasticity, one month after the pond of Folgosinho had been cleaned in spring 2014, we only found five adult Bosca's newts (vs. 228 in 2011) and no overwintering midwife toad larvae (vs. 126 in 2011). This is similar to the northern leopard frog (*Lithobates pipiens*) populations in Saskatchewan (Canada), that have failed to recover after a complex interaction of ecological mechanisms (including mortality by ranavirosis) that precipitated a dramatic collapse (Schock & Bollinger, 2005).

The effects of long-term and frequent CMTV-induced die-offs on natural populations of a susceptible host (here the Bosca's newt) are thus revealed by our

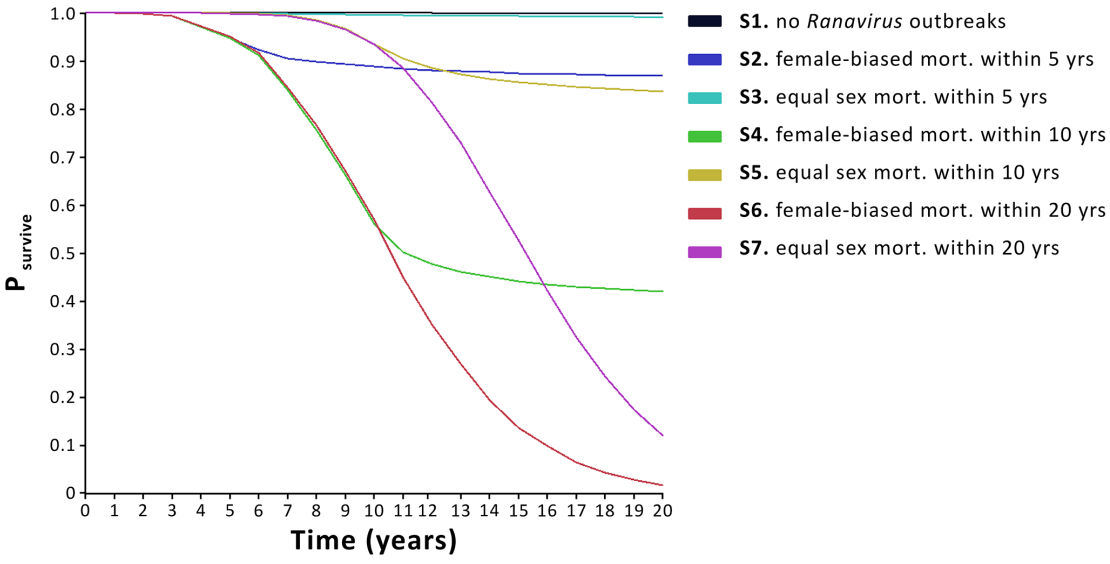

Figure 6. Probability of survival (proportion of iterations in which the population is not extinct) of Bosca's newt (*Lissotriton boscai*) population at different disease scenarios in VORTEX.

demographic evidence and modelling data, suggesting that disease reduces effective population size by reducing the number of breeding adults, which may thus lead to a loss of genetic variability (disease-induced bottleneck; see Fisher, 1930). Host life history may be crucial to maintain a lethal pathogen in the population, as proposed by Brunner *et al*. (2004) in their ambystomatid system. However, here we provide a further step to illustrate how a particular host phenology may facilitate a greater impact of the virus on the population.

5.4.1. Host phenology and pathogen seasonality

The development of clinical ranavirosis can be mediated by environmental factors such as temperature, which is critical for viral replication rate but also affects host susceptibility (Rojas *et al*., 2005; Gray *et al*., 2007). As reported in other systems, epidemics can occur in the late summer or early autumn (e.g. Pfennig *et al*., 1991; see also Gray *et al*., 2009). During late springtime the disease levels drop and virus DNA becomes undetectable with no mortality observed. This seems to happen gradually between winter and early spring.

Seasonal cycles of infectious diseases have been attributed to the behaviour and natural history of the host (e.g. poliomyelitis and measles in humans; see Dowell, 2001), but our data suggests a different angle: environment also affects the phenology of amphibians (the hosts in our system). We observed a correspondence between the seasonality of the pathogen and a phenological window of the host (i.e. biased number of females occurring in water late in the season), making the host more vulnerable to the virus. This seems to be a coincidence and not a pathogen strategy since other previously mentioned factors (biotic and abiotic; Dowell, 2001; Rojas *et al*., 2005; Gray *et al.*, 2007) are known to play a stronger role on seasonality.

Plasticity in phenotypic and life-history traits is not unusual in amphibians and has been frequently reported in caudates (e.g., Hanken & Wake, 1993). However, given the high virulence of this pathogen, the impact on the demographics of the population may be greater if there is a preference for time of year, affecting mainly the females. Previous studies suggest that if the host fails to adapt quickly enough to a novel pathogen or the pathogen fails to evolve lower virulence, the threat of host extinction remains (Vander Wal *et al*., 2014).

5.4.2. Pathogen impact on sex ratio

Ponds may experience epidemics several years in a row, with outbreaks leading to mortality of most larvae in each year (Brunner *et al*., 2004). This was also recorded in our system

where (late) summer outbreaks caused massive mortality of *L. boscai* larvae. Mortality of larvae might be underestimated since individuals are difficult to detect because they are small, decompose quickly, or are scavenged.

Infection of the larval stages is not new (Chinchar *et al*., 2011) since it occurs at a time when densities suddenly increase. However, the outbreak peak may lag behind the peak density due to the viral incubation period (Chinchar *et al*., 2011). This gives time for the adults (first the males) to abandon the pond and explore the terrestrial environment, leaving behind larvae and some adults (mostly females in our system) that have a longer aquatic phase. This explains the high mortality observed in females of the Bosca's newt population, fitting the host-encounter filter concept of Combes (2001): the host-encounter filter tends to exclude host individuals which a pathogen/ parasite cannot encounter due to behavioural or ecological reasons.

One can argue that natural populations of newts present a great deal of variation in sex ratios between years with some populations (of few species) showing males outnumbering females (Arntzen, 2002). A proportion of females skipping annual breeding opportunities could explain this (Arntzen, 2002), however, equal numbers of males and females are generally expected (Díaz-Paniagua & Mateo, 1999). In our case, the initial biased operational sex ratio towards a slightly greater abundance of females (data from Spring 2011) derives from the phenology of this population: the sampling period coincided with the end of the reproductive period when male newts are less active or are leaving their aquatic habitats. This same scenario was observed in the control pond, where this balanced sex ratio was maintained across years. On the other hand, a new trend rapidly emerged after the first outbreaks of ranavirosis in Folgosinho, with a complete reversal of the sex ratio, becoming heavily male-biased in both spring and autumn seasons. The drastic shift and its perpetuation alongside with annual outbreaks suggest a strong correlation.

Additionally, we didn't find a gender-specific susceptibility profile in response to the ranaviral infections since, upon arrival at the ponds, males were also infected and died from ranavirosis. This is a characteristic of the populations found in Serra da Estrela distributed across this altitudinal range. The same cannot be extrapolated to other populations of Bosca's newt, since their behaviour and the time that males and females spend in the water varies with local climatic conditions (see Caetano & Leclair, 1999). However, it is interesting to note that during the first outbreak of ranavirosis described for Portugal in 1998 at the Peneda-Gerês National Park, a similar pattern was also observed in the marbled newt (*Triturus marmoratus*) population, in which the authors stated that "females especially appeared to be affected" (Froufe *et al*., 1999).

Sex-biased mortality has been reported across different vertebrate groups (particularly birds and mammals) with a great diversity of complex mechanisms that range from species physiology (e.g., Müller *et al*., 2005) and behaviour (e.g., Sperry & Weatherhead, 2009), to external environmental factors. However, it is assumed that, in most cases, pathogens would have detrimental effects at the host population level, particularly when targeting females, which had never been demonstrated until now. In any case, induced mortality skewed towards females in vertebrates seems to lead to increased effects on population demography accelerating local decline, which was has demonstrated by modelling (Grüebler *et al*., 2008).

5.4.3. Disease outbreaks and population persistence

Our results show that a sex-biased mortality may easily exacerbate the unbalanced sex ratio and increase the extinction risk of the population. Nonetheless, despite the importance of pathogens in wild populations, little attention has been given to host-pathogen dynamics in PVAs (Gerber *et al*., 2005). Although there is no evidence from natural populations that *Ranavirus* can decrease its virulence in the host species, this evolutionary dynamic has been shown in experimental conditions through mutations and recombination events (Ebert, 1998), particularly in *Ranavirus* (Chen *et al*., 2011). This was considered in our projections where the outbreak scenarios were modelled to stop after 5 or 10 years.

Five years of annual ranavirosis outbreaks (as observed in our system) may account for a transient emergence event, which is one of the two behavioural strategies suggested by Teacher *et al*. (2010). In this case, if die-offs stop, the population would have good chances of recovery, but take longer if female-biased mortality was happening. The other proposed viral behaviour would be its mid- to long-term persistence with recurrent mortality events (Teacher *et al*., 2010). With that in mind, our projections assume the possibility of eventual conservation intervention/ mitigation actions stopping the mortality after 10 years, or a scenario of indefinite recurrent outbreaks for at least an additional 10 years. We obtained a median risk of extinction of 11 to 16 years if outbreaks take place yearly for at least 10 years, but with an extinction risk of over 88% if no human intervention is in place. There are other suitable hosts in this system that can maintain the virus indefinitely in the system. Apart from other sensitive dying species, the fire salamander (*Salamandra salamandra*) may potentially act as a silent host (see Chapter V).

The key finding of our modelling is that female-biased mortality does not exacerbate the population decline in the short-term, but will do so in the mid- to long-

term. In any case, the impact of biased mortality will be immediately felt during an eventual recovery of the population after potential ceasing of mass mortality events. This hypothesis is supported by the different projections that show a higher likelihood of population recovery with a faster growth. This raises special concerns from the population management point of view, but also establishes a window of opportunity where efforts to mitigate the impact may result in successful recovery of a population.

5.4.4. Ranavirus ecology and host species conservation

For infectious disease systems, it is generally expected that co-evolution of an emerging pathogen and its host will not result in extirpation of the host (Paterson *et al*., 2010). This process implies a density-dependent mechanism that leads to the loss of pathogen or attenuation of its virulence before the host population is driven to extinction (McCallum & Dobson, 1995). The model has been suggested in the tiger salamander-ATV *Ranavirus* system through phylogenetic concordance (Storfer *et al*., 2007) but also because tiger salamanders are commonly found in the absence of other amphibians and epidemics are common (Brunner *et al*., 2004), involving reciprocal fitness costs. Using a different single host-parasite system, Duffy *et al*. (2007) also showed a rapid evolution of *Daphnia dentifera* and its parasite *Metschnikowia bicuspidata*, where hosts from lakes with recent epidemics were more resistant to infection and had less variance in susceptibility.

However, in our multi-host community system, as well as in other CMTV-affected sites in Iberia (see Price *et al*., 2014), it seems that a more complex mechanism is occurring. Given the broad host range and the chance to maintain pathogen circulation in the environment with transmission taking place even at the lowest density (through a density-independent manner), extinction of the population is possible. The density effect threshold is virtually eliminated, increasing the chance of pathogen-induced host extinction. To reinforce that, this pathogen has been detected year-round in these newts and also on other species of the Serra da Estrela amphibian assemblage (Chapter IV).

Only a few parasites and pathogens are known to have the ability to disrupt freshwater ecosystems as much as ranaviruses (Gray & Miller, 2013). We note that the impact of a pathogen may be greater or smaller depending on the phenology of its host, within the same species. But a continuous long term monitoring scheme is necessary to evaluate infection trends on the different ectothermic vertebrate groups (fish, amphibians and reptiles) and identify potential drivers of infection in host communities.

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Chapter VI

General Discussion

General Discussion

This thesis documents the impact of two multi-host virulent pathogens on the same amphibian assemblage, focusing mainly on disease emergence, population declines, trends and viability. In an attempt to fill a knowledge gap, I tried to understand differential host susceptibility to virulent pathogens and the long-term outcome of host-pathogen dynamics using data generated from both field and laboratory research. A multidisciplinary approach was favoured, using different tools in order to understand the disease-host population dynamics, so that the results obtained can be applied to effective population management and conservation.

The amphibian chytrid fungus was found to be involved in the decline of midwife toads in Serra da Estrela, a mountainous biodiversity hotspot in northern central Portugal. This generalist pathogen had differential effects across taxonomic groups but also within the same species across an altitudinal gradient. I describe the asynchronous emergence of a second pathogen (*Ranavirus*) within that area affecting a broader range of species, life stages and sexes, leading to annual mortality events in virtually all the hosts. I also show that the natural history and particular behaviour of certain species have the ability to mediate the impact of both pathogens in this amphibian assemblage.

Pathogens can have strong effects on species interactions, playing an important role in the dynamics of community structure with potential dramatic consequences. This may lead to reduced population densities or even cause extinctions (Dobson & Hudson 1986; Kiesecker & Blaustein, 1999; Daszak *et al*., 2000; Bosch & Rincón, 2008). The impact of infectious diseases on amphibian populations with associated declines and extinctions has raised pathogens to a state of global importance. As a consequence, research in this field has increased in recent years, but with limited applicable outcomes in terms of conservation (Garner *et al*., 2013).

6.1. Amphibian population collapses in the Iberian Peninsula

Pathogens that follow multiple host strategies seem to have the capacity to produce and maintain high levels of genetic diversity or a biological cycle/ecology that provides them the opportunities for cross-species transmission (Woolhouse *et al*., 2001; Pulliam & Dushoff,

2009). This seems to be the case for both the amphibian chytrid fungus and *Ranavirus*, as both are able to infect and use several species as hosts (Fisher *et al*., 2012; Chinchar & Waltzek, 2014). Their impact has been shown to vary across species but is also influenced by environmental factors (Walker *et al*., 2010; Chinchar & Waltzek, 2014). Genetic diversity within the pathogen seems to play a significant role with different amphibian species showing different outcomes when in contact with different strains of *Bd* (Farrer *et al*., 2011) and *Ranavirus* (Duffus *et al*., 2014).

At Serra da Estrela, where both pathogens are now known to be present, we observed an unusual pattern where chytrid-mediated declines of *Alytes obstetricans* were followed by the later emergence of *Ranavirus* that led to mass mortalities of the same species across the altitudinal range. According to reports from the 1990's, *A. obstetricans* used to be one of the most abundant and common species of amphibians in that mountain area. In certain freshwater sites at the Planalto Superior region it was possible to count thousands of large tadpoles (Brito-e-Abreu *et al*., 1994). Within a few years, *Bd* caused in a decline of 67% in this species range, with a much lower abundance of larvae and breeding currently limited to 16% of the original breeding sites (Chapter II; Rosa *et al*., 2013). This high mortality was significantly more accentuated in the higher elevation areas, a trend that has also been observed in populations of *A. obstetricans* in other high-elevation sites in other European countries (Walker *et al*., 2010). In contrast, it was still possible to find abundant populations of midwife toads in lower elevation sites in the spring of 2011, such as Folgosinho. Later that year, the emergence of a second hyper-virulent pathogen extended to this population of toads, and affected all amphibian species present (Chapter IV).

Ranavirus was first detected in Serra da Estrela in the summer of 2011 and is now relatively widespread throughout the Natural Park. Infection associated with this disease and mass mortality was later observed in autumn (November), when 92.3% of the Bosca's newts found in the water at a specific site were dead. The same scenario was then repeated annually at about the same time of the year (late summer / early autumn) with numerous dead and dying caudates and anuran amphibians (adults and larvae) encountered and testing positive for *Ranavirus* (Chapter IV).

Data recorded prior to the first outbreak, as well as the existence of a comparative site with similar geo-climatic features where mortality due to *Ranavirus* was never recorded, allowed the determination of population trends of several hosts during the outbreaks. Where *Ranavirus* outbreaks happened annually, populations of all the species faced a steep decline, with the exception of the fire salamander (*Salamandra salamandra*),

where larvae had always been found in low densities (Chapter III and IV), matching with previous records from the 1990's (Brito-e-Abreu *et al*., 1994).

Interestingly, while highly pathogenic lineages of *Bd* (e.g. GPL) seem to have a smaller range of hosts that are being affected and declining due to pathogen exposure in natural conditions, *Ranavirus* (such as the hyper-virulent CMTV-like strains) appear more severe, leading to sickness and high mortality, virtually, across all the amphibian species present in a certain pond (Price *et al*., 2014; Chapter IV).

Chytridiomycosis is thought to be the most significant disease currently affecting biodiversity of vertebrates (Skerratt *et al*., 2007). However, the results on CMTV-like *Ranavirus* raise concerns for management and conservation of wild populations, compounded by the fact that (unlike the *Bd*) there is still no way to mitigate or clear the disease, even in captive individuals.

6.2. Differential susceptibility to a virulent pathogen

Pathogens such as viruses, fungi and/or bacteria can be highly lethal to naive hosts leading to mass die-offs and even local extinctions (Lesbarrères *et al*., 2011; Fisher *et al*., 2012). However, while some species seem to disappear completely following a *Bd* outbreak, other species persist without being (apparently) affected (e.g. Retallick *et al*., 2004; Newel *et al*., 2013). These differences in response may have a genetic origin, both at an individual- and at a population-level (Addis *et al*., 2015), or be mediated by environmental factors (Filotas & Hajek, 2004; Walker *et al*., 2010). As an example of the latter, the hyper-virulent lineage of the amphibian chytrid fungus (*Bd*GPL) has been linked to population crashes of common midwife toads (*A. obstetricans*) in high elevation areas such as Serra da Estrela and Peñalara (Spain) (Bosch *et al*., 2001; Rosa *et al*., 2013). Although *Alytes* has been regarded as a very sensitive host, this largely contrasts to the reality across most of the species range, where populations might be infected but no declines have been recorded, even in other higher elevation places such as the Pyrenees (Walker *et al*., 2010; Böll *et al*., 2012; Schmeller *et al*., 2014).

Yet, and contrasting with the decline of midwife toads, *S. salamandra* populations showed no significant variation in abundance in recent years (Chapters III & IV), which may suggest higher resistance when exposed to *Bd* and *Ranavirus*. However, since it was still possible to detect ranaviral infection in a few individuals, the species may otherwise act as a silent host for this virus. When looking at the host-pathogen interaction in the case of *Bd* exposure, the final outcome seems similar in the Serra da Estrela system with no *Bd*-

positive salamander or *Bd*-mediated mortality ever been recorded at any of the monitored sites (Chapter III). However, the salamander-pathogen dynamics appears to be different and explained by other mechanisms (see 6.3).

Fire salamanders may be extremely efficient at blocking epidermal colonization by *Bd*, preventing infection and/or making it easier to clear, as observed in other caudate species (Pasmans *et al*., 2013). Nevertheless, sub-lethal effects exist in exposed individuals, indicating that successful immune responses to *Bd* come at a cost. But in face of what has been reported in other systems, resistance seems not to be a consistent trait across fire salamander populations (Chapter III).

Peculiar aspects of host behaviour, natural history, life-cycle and/ or phenology have not been often considered in epidemiological studies. This approach was addressed in Chapters III and V, in an attempt to both explain differences in exposure outcomes across different populations and to understand how host phenology may mediate the impact of a pathogen.

6.3. Natural history and behaviour as mediators of host-pathogen interaction outcomes

Differences in host susceptibility influence the impact of the disease, and drive ecological phenomena such as pathogen amplification or dilution (Schmidt & Ostfeld 2001; Keesing *et al*. 2006). It has also been suggested that certain life-history strategies may increase (or decrease) rates of pathogen transmission or growth (Searle *et al*., 2011). However, few studies have taken account of host behaviour in the regulation of disease impacts.

The failure to detect sick and/ or chytrid-infected *S. salamandra* in Serra da Estrela contrasts with the reality faced in Guadarrama and the Pyrenees, where larvae often test positive for *Bd* (Bosch & Martínez-Solano, 2006). As discussed in Chapter III, life history traits and environmental constraints might be driving these differences, with larvae in the higher elevation sites (Guadarrama and the Pyrenees) often overwintering and thus experiencing an extended period of contact with the pathogen in the water (Medina *et al.*, in press). This prolonged exposure may then increase the chances of infection and support the idea that the impact of a virulent pathogen can be mediated by host life history. The study I developed on the Bosca's newt population in Serra da Estrela confirms this: the particular phenology of this host amplifies the lethal effects of the annual exposure to *Ranavirus* (Chapter V). A sex-biased mortality exacerbates the unbalanced sex ratio and increases the extinction risk of the population.

Density-dependent transmission only happens until the host population drops to a certain threshold size, rarely driving the host to extinction (Grenfell & Dobson, 1995, Hudson *et al*., 2002). For generalist multi-host pathogens, the threshold density effect is virtually eliminated, increasing the chance of pathogen-induced host extinction (de Castro & Bolker, 2005; Greer *et al*., 2008; Brunner *et al*., 2007), which seems to be the most likely explanation for *Bd* and *Ranavirus* effects at Serra da Estrela. The presence of suitable reservoir hosts (as in my natural system) can thus lower the pathogen's threshold density and lead to local (population) extinction (McCallum & Dobson, 1995; Woodroffe, 1999). Taking that into account, the observed female-biased mortality seems likely not to exacerbate the population decline in the short-term, but will do it on a mid- to long- term (Chapter V). In any case, the impact of sex-biased mortality will be immediately felt during an eventual recovery of the population after potential ceasing of mass mortality events, where models of sex-balanced mortality show a much faster recovery (Chapter V).

6.4. Final considerations

The data presented provide significant evidence that *B. dendrobatidis*-mediated declines and extinctions occur for an ecologically predisposed group of species, whereas *Ranavirus* CMTV-like strains seem to have a broader impact across amphibian species.

Integrative approaches (combining molecular techniques, behaviour, field surveys, etc) provide new lines for species conservation efforts as well as facilitating further understanding of epidemiology. The sequencing of 29 strains of *Bd* (Rosenblum *et al*., 2013) showed a huge genetic variability previously unknown, which can be a clue to a new question: what makes certain pathogen strains/ lineages so deadly? These new genetic data will permit analyses of recent environmental changes that may have played an important role in increasing pathogen virulence. Genomics is a path that only now is starting to be unraveled. Its application to natural populations may help to understand the variation in the adaptive genetic diversity and of the genes involved in resistance and susceptibility to diseases and other environmental stressors (Storfer *et al*., 2009).

Good surveillance and monitoring are key steps for effective management and conservation of wild populations. Long-term records allow a more efficient evaluation of their dynamics, enabling natural fluctuations to be distinguished from unusual declines (Pechmann *et al*., 1991; Meyer *et al*., 1998). In addition, continuous monitoring of the health status of wild populations becomes crucial for subclinical detection of disease and timely adoption of mitigation measures (Obon *et al*., 2013).

Development and implementation of mitigation strategies is one essential component of population management. However, most of the existing disease mitigation protocols are still in early experimental stages (Woodhams *et al*., 2011) and so far are not transferable across such different pathogens as *Bd* and *Ranavirus*. Prophylactic or remedial treatments for chytridiomycosis have been developed showing optimistic results in laboratory setups, from antifugal baths (e.g., itraconazole or voriconazole solutions; Garner *et al*., 2009; Martel *et al*., 2011) to heat therapy (e.g., Chatfield & Richards-Zawacki, 2011), antimicrobial peptides and bacteria (Bletz *et al*., 2013; but see Antwis *et al*., 2015). On another front, environmental manipulation can be implemented to manage decrease infection rates and burdens and hence improve host survival (Scheele *et al*., 2014). In addition to suggested protocols such as introduction of *Bd* inhibitors or exclusion of *Bd* reservoir host species (Woodhams *et al*., 2011), the characterization of planktonic microorganism communities in relation to *Bd* presence and absence may also lead to an effective landscape level mitigation strategy (Schmeller *et al*., 2014). Bioaugmentation of these communities with *Bd*-consuming microfauna may inhibit the colonization by *Bd* of new habitats.

Regarding ranavirosis the same cannot be said since only recently has it been identified as a conservation issue, even if it was occasionally causing mass die-offs among commercial fish stocks (Whittington *et al*., 2010). Thus, medical treatments and/ or prophylactics are currently unavailable as well as protocols for the wild populations. Artificial selection has been successful improving resistance to viral pathogens in aquaculture species such as shrimp (e.g., Argue *et al*., 2002) and several fish species (e.g., Henryon *et al*., 2005; Kjøglum *et al*., 2008), which can be a way to deal with *Ranavirus*. As for chytridiomycosis, interventions should target life history stages most affected by disease or at high risk of pathogen exposure. In any case, common management priorities seem to rely on halting pathogen spread, preventing introduction in new geographic areas and populations naïve to those pathogens or particular strains (Whittington *et al*., 2010).

Successful disease mitigation should then be context specific with epidemiologically informed strategies to manage affected populations by decreasing pathogenicity and host susceptibility. Sustainable conservation of wild amphibian assemblages is dependent on long-term population persistence and co-evolution with these lethal pathogens.

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Chapter VII

Appendices

Additional publications

7.1. Selected peer reviewed (* sharing first co-authorship)

Bielby J, Fisher MC, Clare FC, **Rosa GM**, Garner TWJ (2015). Host species vary in infection probability, sub-lethal effects, and costs of immune response when exposed to an amphibian parasite. *Scientific Reports* 5:10828. 133

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7.2. Natural history and first records peer reviewed

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7.3. Books and chapters

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SCIENTIFIC REPORTS

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Host species vary in infection probability, sub-lethal effects, and costs of immune response when exposed to an amphibian parasite

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The amphibian parasite *Batrachochytrium dendrobatidis* (*Bd*) is regarded as an extreme generalist, infecting over 500 species, but amongst these hosts there exists a great deal of variation in the susceptibility to and the costs of parasite exposure. We use two infection experiments to determine whether inter-specific variation in the sublethal and lethal effects of parasite exposure exist in two host species. We then tested the relative roles of host density and diversity on infection probability of a focal susceptible host. Our results show significant heterogeneity in host species response to parasite exposure, and that both lethal and sub-lethal costs exist in individuals that are able to resist infection, indicating that successful immune response to infection comes at a cost. Further, we show that increasing host density significantly increased the likelihood of susceptible individuals becoming infected with *Bd* irrespective of host diversity and variation in host susceptibility. These results suggest that populations of resistant species are likely to suffer ill-effects of exposure to *Bd* regardless of their infection status, and that at the stage of initial infection there was no support for the dilution of transmission events, in contrast to other studies that focus on subsequent transmission of infection.

The majority of parasites infect multiple hosts[1], but they do so to varying degrees; even the most generalist parasites exhibit considerable variation in how frequently and heavily they infect species within their host-base[2]. In some host species many individuals will be infected, and these infections may be severe with a high number of individual parasites per host, whereas in other host species infections will be less common and infections will tend to be lighter. This inter-specific variation in infection frequency and severity is not the only way species respond differently to the same parasite; it is mirrored in other aspects of host-parasite dynamics, such as differences in the consequences of parasite exposure, and the roles different host species play in transmission of infection within a community.

Different host species exhibit a great deal of variation in the consequences of parasite exposure. Hosts may experience significant inter-specific variation in mortality rates and population trajectories as a result of parasite exposure[2], but variation may be more subtle and still have an effect on individual hosts, their populations and the communities in which they live. The sub-lethal effects of parasite exposure can be considerable at the level of the individual and may scale up to the level of the population, yet they are often overlooked in studies of wildlife disease. Sub-lethal infections can reduce fecundity, increase

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Widespread presence of the pathogenic fungus *Batrachochytrium dendrobatidis* in wild amphibian communities in Madagascar

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Amphibian chytridiomycosis, an emerging infectious disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has been a significant driver of amphibian declines. While globally widespread, *Bd* had not yet been reported from within Madagascar. We document surveys conducted across the country between 2005 and 2014, showing *Bd*'s first record in 2010. Subsequently, *Bd* was detected in multiple areas, with prevalence reaching up to 100%. Detection of *Bd* appears to be associated with mid to high elevation sites and to have a seasonal pattern, with greater detectability during the dry season. Lineage-based PCR was performed on a subset of samples. While some did not amplify with any lineage probe, when a positive signal was observed, samples were most similar to the Global Panzootic Lineage (*Bd*GPL). These results may suggest that *Bd* arrived recently, but do not exclude the existence of a previously undetected endemic *Bd* genotype. Representatives of all native anuran families have tested *Bd*-positive, and exposure trials confirm infection by *Bd* is possible. *Bd*'s presence could pose significant threats to Madagascar's unique "megadiverse" amphibians.

Amphibian population declines and extinctions are occurring at unprecedented rates[1]. Multiple anthropogenic factors including habitat destruction and alteration, introduction of alien species and overexploitation are linked to the global declines of amphibians. Chytridiomycosis, an emerging infectious disease caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*), is also recognized as playing a significant role in the rapid declines and extinctions of amphibians around the world[2,3]. *Bd* has been detected in over 500 species worldwide (http://www.bd-maps.net/), and at least 200 species have declined as a result of chytrid

PLOS ONE

RESEARCH ARTICLE

Phylogeny and Differentiation of Reptilian and Amphibian Ranaviruses Detected in Europe

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Data Availability Statement: All sequence data have been made available in GenBank, and the accession numbers are provided in the paper. All other relevant data are within the paper and its supporting information files.

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Abstract

Ranaviruses in amphibians and fish are considered emerging pathogens and several isolates have been extensively characterized in different studies. Ranaviruses have also been detected in reptiles with increasing frequency, but the role of reptilian hosts is still unclear and only limited sequence data has been provided. In this study, we characterized a number of ranaviruses detected in wild and captive animals in Europe based on sequence data from six genomic regions (major capsid protein (MCP), DNA polymerase (DNApol), ribonucleoside diphosphate reductase alpha and beta subunit-like proteins (RNR-α and -β), viral homolog of the alpha subunit of eukaryotic initiation factor 2, eIF-2α (*v*IF-2α) genes and microsatellite region). A total of ten different isolates from reptiles (tortoises, lizards, and a snake) and four ranaviruses from amphibians (anurans, urodeles) were included in the study. Furthermore, the complete genome sequences of three reptilian isolates were determined and a new PCR for rapid classification of the different variants of the genomic arrangement was developed. All ranaviruses showed slight variations on the partial nucleotide sequences from the different genomic regions (92.6–100%). Some very similar isolates could be distinguished by the size of the band from the microsatellite region. Three of the lizard isolates had a truncated *v*IF-2α gene; the other ranaviruses had full-length genes. In the phylogenetic analyses of concatenated sequences from different genes (3223 nt/10287 aa), the reptilian ranaviruses were often more closely related to amphibian ranaviruses than to each other, and most clustered together with previously detected

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A new phytotelmic species of *Platypelis* (Microhylidae: Cophylinae) from the Betampona Reserve, eastern Madagascar

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Abstract. We describe a new arboreal and diminutive species of the genus *Platypelis* from the Réserve Naturelle Intégrale N. 1 de Betampona, one of the last low-altitude rainforest fragments of eastern Madagascar. *P. karenae* sp. nov. is a phytotelmic species, living among leaves of *Pandanus* spp. and those of a herbaceous plant of the genus *Crinum*. Amongst species of comparable size, the new species is most similar to *P. tetra*, with which it shares a similar life history of occupying leaf axils of phytotelms. Phylogenetically, *P. karenae* is sister to *P. tuberifera* yet differentiated by a high level of genetic divergence (>7% p-distance for the analysed fragment of the 16S rRNA gene), its distinctly smaller size, acoustic repertoire, and colour pattern. The mitochondrial, nuclear, bioacoustic, and morphological data all independently support the validity of this new species.

Key words. Amphibia, Anura, new species, miniaturisation, *P. karenae* sp. n.

Introduction

Frogs of the highly diverse family Microhylidae are distributed throughout the tropical forests of the globe, and exhibit a wide variety of habitat adaptations and morphological variations. The cophyline genus *Platypelis* is endemic to Madagascar, and currently includes 12 described species and at least other six confirmed candidate species (Glaw & Vences 2007, Vieites et al. 2009, Perl et al. 2014). The species of this genus are small to medium-sized frogs (16–40 mm), with the exception of *P. grandis,* which reaches up to 105 mm in snout–vent length (SVL) and is one of the largest microhylid frogs worldwide (Glaw & Vences 2007).

All known *Platypelis* species are arboreal, with enlarged finger and toe disks, and breed in phytotelmata or water-filled bamboo internodes or tree holes. As far as is known, the male guards the eggs and non-feeding tadpoles until their metamorphosis (Blommers-Schlösser 1975). In terms of biogeography, the genus is mainly distributed in the rainforests of northern and northeastern Madagascar, where several species have recently been discovered and from where several confirmed candidate species are currently known and still need to be formally described (Andreone et al. 2005, Glaw & Vences 2007, Rakotoarison et al. 2012, Rosa et al. 2012). No species of *Platypelis* has yet been found in the arid western regions and the deciduous forests of Madagascar, presumably because of their need for continuously high humidity (Mercurio et al. 2008, Bora et al. 2010) and appropriate phytotelmic breeding sites.

Typically, *Platypelis* males call perched on leaves or branches and inflate a single, quite expandable, subgular vocal sac. Their vocalizations show a rather similar temporal succession, consisting of single and monotonous notes repeated in almost endless series, as in most cophylines (Glaw & Vences 2007). Notable exceptions to this pattern

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ORIGINAL PAPER

Case solved: presence of toxin-secreting oral glands in the lamprophiid snake *Mimophis mahfalensis* (Grandidier, 1867) from Madagascar

Gonçalo M. Rosa · Renaud Boistel · Ezio Campantico · Brigitte Gillet · Paolo Eusebio Bergò · Franco Andreone

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Abstract The monotypic *Mimophis mahfalensis* is an opisthoglyphous snake endemic of Madagascar. The goal of this study is to clarify the presence of toxin-secreting oral glands through a multidisciplinary approach. We thus provide data on the internal anatomy of the head of *M. mahfalensis*' and demonstrate the presence of Duvernoy's glands. Furthermore, we refer to two cases of human bitten by this species and compared this with the reported case of mild envenomation mentioned by Domergue (Arch Inst Pasteur Madagascar 56(1):299–311, 1989). Symptoms included brief slight local pain and minor bleeding.

Keywords Duvernoy's gland · Madagascar · *Mimophis mahfalensis* · Opisthoglyph

Introduction

Within the speciose herpetofauna of Madagascar, with currently more than 290 species of amphibians and around 370 species of reptiles (Glaw and Vences 2007), snakes deserve particular attention. Malagasy boids are a clade sister to the genus *Calabaria* from Africa, showing a peculiar relationship with other constrictor. This relationship is better reflected by current geographical distributions and tectonic history than by the morphological characters (Noonan and Chippindale 2006; Crottini et al. 2012), while blind snakes of Madagascar form a clade that split off earlier than other scolecophidians (Vidal et al. 2010). In addition, Malagasy snakes are also notable in that there are no species really dangerous to humans. Moreover, there are no solenoglyphous or proteroglyphous species, except for rare reports of the occurrence of the marine sea snakes of the genera *Enhydrina* and *Pelamis* (Glaw and Vences 2007). Most terrestrial snakes that were formerly

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RESEARCH ARTICLE

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A new species of the *Boophis rappiodes* group (Anura, Mantellidae) from the Sahamalaza Peninsula, northwest Madagascar, with acoustic monitoring of its nocturnal calling activity

Samuel G. Penny[1,2], Franco Andreone[3], Angelica Crottini[4], Marc W. Holderied[1], Lovasoa Sylviane Rakotozafy[5], Christoph Schwitzer[2], Gonçalo M. Rosa[6,7,8]

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Abstract

A new species of treefrog of the *Boophis rappiodes* group (Anura, Mantellidae) is described from the Sahamalaza – Iles Radama National Park in northwest Madagascar. This new species is green in colour with bright red speckling across its head and dorsum; similar in morphology to other species of this group including: *B. bottae*, *B. rappiodes*, *B. erythrodactylus* and *B. tasymena*. The new species can be distinguished by its advertisement call and by a genetic divergence of more than 4.9% in the analysed mitochondrial

Zoo Biology 32: 457–462 (2013)

BRIEF REPORT

Differential Effects of Dietary Protein on Early Life-History and Morphological Traits in Natterjack Toad (*Epidalea calamita*) Tadpoles Reared in Captivity

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The production of high quality amphibian larvae through optimal diets is a critical component of amphibian conservation breeding programs. Larval period, survival, body weight and total length are frequently used as metrics of adequate nutrition. However, the effects of nutrition on tadpole and metamorph morphology are rarely tested in detail. In the present study, we analyzed the most common metrics and six other larval and post-metamorphic morphological traits in natterjack toads (*Epidalea calamita*) fed with three different commercial fish diets, varying in protein content (32.0%, 38.3%, and 46.2%). Our results suggest that early life-history (tadpole growth, development, and survival) and morphological traits of *E. calamita* tadpoles are differentially affected by the percentage of dietary protein. As protein content increased, tadpoles exhibited larger bodies along with shorter tail fins; however, with no significant differences in total length. Larval period was similar across treatments but mortality was lower in high-protein diet. At high-protein diets the metamorphs revealed significantly longer bodies, and wider heads and hind legs, but there was no significant difference in the average weight across all dietary treatments. Based on our results, feed containing 46.2% protein promotes growth, development and survival of *E. calamita* tadpoles better than either of the other two feeds tested. The use of other body measures beyond weight, tadpole total length, and snout-vent length in studies of amphibian nutrition in captivity may assist the selection of appropriate diets to optimize tadpole survival and metamorph fitness. Zoo Biol. 32:457–462, 2013.

Keywords: captive rearing; *Epidalea calamita*; metamorphosis; morphology; protein; tadpoles

INTRODUCTION

Nutrition is one of the main husbandry issues when rearing amphibian larvae, particularly within conservation breeding programs [Browne and Zippel, 2007]. Despite its crucial importance, the knowledge of amphibian nutritional requirements among researchers and zoo staff does not reflect the increasing number of species that require captive breeding programs (see Altig et al. [2007] for details regarding feeding ecology of amphibian larvae) and only comprises a minor fraction of the current literature on amphibian captive rearing [Browne et al., 2011].

Dietary protein plays an important role in the growth and development of amphibian larvae [Kupferberg, 1997] and, therefore, a knowledge of the dietary protein requirements of any species is essential to optimize its health and development. Most of the studies published on amphibian nutrition are directed to those few species used in frog farming [e.g., Martínez et al., 1993, 1994; Carmona-Osalde et al., 1996; Sretarugsa et al., 1997]. However, in conservation breeding programs, larval husbandry aims to improve long-term fitness

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Evidence of chytrid-mediated population declines in common midwife toad in Serra da Estrela, Portugal

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Keywords
Batrachochytrium dendrobatidis; chytridiomycosis; Iberian Peninsula; outbreak; Serra da Estrela

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Abstract

The emergence of a novel infectious disease, chytridiomycosis, is now widely recognized as a major cause of amphibian declines and biodiversity loss across local and global scales. Amphibian mortalities caused by the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*) were first recorded in Iberia, Europe over a decade ago. In August 2009, hundreds of post-metamorphic common midwife toads (*Alytes obstetricans*) were found dead in the water and margins of a pond in the Serra da Estrela Natural Park, north-central Portugal. Histological and genetic analyses confirmed their infection with *Bd*. Given the likelihood of a new outbreak of chytridiomycosis, we evaluated the possible impacts of this disease on populations of *A. obstetricans* within the Park by conducting field surveys during 2010 and 2011. We compared the present distribution and abundance of *A. obstetricans* with historical records, and quantified the present prevalence and intensity of infection by *Bd*. Results showed that (1) *A. obstetricans* disappeared from 67% of the 1×1 km squares where it was recorded in the past, (2) breeding is currently limited to 16% of the confirmed breeding sites in the past, and that (3) larvae are now less abundant, as well as are highly infected by *Bd* in the remaining sites. These effects were most pronounced at altitudes above 1200 m. Our findings suggest that an outbreak of chytridiomycosis is responsible for the rapid decline of *A. obstetricans* in Serra da Estrela, and we believe that urgent conservation measures are needed to prevent local extinction of the species.

Introduction

Amphibian species are declining at an alarming rate on a global scale and are currently considered among the most threatened groups of vertebrates, with up to one-third of species threatened by extinction (Stuart *et al.*, 2004). The global decline of amphibians is caused by multiple factors, the most prominent being habitat loss, invasion of non-native species, overexploitation and outbreaks of infectious diseases. It is now widely recognized that the emergence of a novel infectious disease, chytridiomycosis, is driving rapid amphibian declines and biodiversity loss at the global scale (Berger *et al.*, 1998; Stuart *et al.*, 2004). The most severe outbreaks of chytridiomycosis have been described from the American Neotropics where the disease is reported to have been responsible for the extinction of about 67% (110 species) of *Atelopus* spp. frogs all across the distribution range of the genus (La Marca *et al.*, 2005) and to have caused the rapid loss of amphibian biodiversity across eight families of frogs and salamanders at El Cope, Panama (Lips *et al.*, 2006). However, severe impacts of chytridiomycosis have also been recorded across diverse tropical, temperate and montane environments, including the Caribbean, South America (particularly the Andean region), Australia, North America and Europe (Fisher, Garner & Walker, 2009*b*; Catenazzi *et al.*, 2011).

The aquatic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is the etiological agent of chytridiomycosis (Fisher *et al.*, 2009*a*) and is known to infect over 508 species of amphibians (http://www.bd-maps.net/). The pathogen produces two key lesions: the loss of sections of keratinized mouthparts in amphibian larvae (Fellers, Green & Longcore, 2001; Knapp & Morgan, 2006), and the proliferation of keratinaceous cells and hyperkeratosis in post-methamorphic individuals (Berger *et al.*, 1998; Kilpatrick, Briggs & Daszak, 2010). The final mechanism by which *Bd* causes morbidity and mortality in amphibians appears to be the disruption of skin integrity and cutaneous function,

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RESEARCH ARTICLE

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Rana iberica (Boulenger, 1879) goes underground: subterranean habitat usage and new insights on natural history

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Abstract

Reports of amphibians exploiting subterranean habitats are common, with salamanders being the most frequent and studied inhabitants. Anurans can occasionally be observed in caves and other subterranean habitats, but in contrast to salamanders, breeding had never been reported in a cave or similar subterranean habitat in Western Europe. Based on observations during visits to a drainage gallery in Serra da Estrela, Portugal, from May 2010 to December 2012, here we document: (i) first report of *Rana iberica* reproduction in cave-like habitat, representing the fourth report of an anuran for the Palearctic ecozone; (ii) oophagic habits of the tadpoles of *R. iberica*; and (iii) *Salamandra salamandra* predation on *R. iberica* larvae. These observations, particularly of *R. iberica*, highlight our lack of knowledge of subterranean ecosystems in the Iberian Peninsula.

Keywords

Subterranean habitats, amphibians, anuran reproduction, larval predation, oophagy

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SPATIAL ECOLOGY OF *SCAPHIOPHRYNE GOTTLEBEI* IN THE CANYONS OF THE ISALO MASSIF, MADAGASCAR

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ABSTRACT: The Rainbow Frog *Scaphiophryne gottlebei* lives within the humid canyons that cross the Isalo Massif, central-southern Madagascar. Knowing that a single haplotype dominates the largest part of its distribution range raised questions about the dispersal ability of the species. We affixed external radio transmitters to 36 individuals of *S. gottlebei* to understand whether the adults of this species actively displaced from the canyon they inhabit. We studied 13 males and 7 females in 2009 and 7 males and 9 females in 2011 over two periods (November–December 2009 and January–February 2011). Study sessions were chosen due to the different meteorological conditions: the first is the beginning of the rainy season, which corresponds to the start of reproduction; and the second being the end of the rainy season, which corresponds to a wetter period during which individuals are more likely to be feeding in order to increase body weight. Our results revealed that there is no significant difference in the activity patterns between sexes and that rain and temperature stimulate the dispersal rate. The distance covered by the individuals did not differ between males and females; the range varied from a few centimeters to approximately 50 m, although two individuals displaced more than 100 m in a single day. These data suggest that individuals of this species are quite phylopatric to the canyons they inhabit. The generalized haplotype sharing observed might then be explained by passive dispersal of larvae and metamorphosed individuals during the intense cyclonic floods.

Key words: Amphibians; Ecology; Radiotelemetry; Rainbow Frog

THE CURRENT COUNT of Madagascar amphibians adds up to more than 280 species, many of which are still waiting to be described. For this reason most research activities have dealt with species descriptions and phylogenetic reconstructions. Comparatively less information is available on the ecological traits and life histories of most species on the island. This is especially true for the species assessed as threatened by the Red List (IUCN, 2010). Thus, an increase in ecological and behavioral studies may be seen as a new challenge for anuran research on the Great Island in forthcoming years in conjunction with the implementation of effective conservation measures such as those listed by the Sahonagasy Action Plan (Andreone et al., 2008a, 2012). In particular, it is essential to gather data on reproductive biology and habitat preferences in order to understand species' ecological needs and sensitivities (Andreone and Luiselli, 2003). This is also important when captive breeding programs are proposed as a possible response action to emerging infectious diseases, such as the lethal amphibian chytrid fungus which could become a dramatic threat for Madagascan frogs if its presence is confirmed (Crottini et al., 2011; Rabemananjara et al., 2011).

Recently we studied the life history and ecology of one of the most threatened and cryptic species of Madagascar, the Rainbow Frog, *Scaphiophryne gottlebei*. Described by Busse and Böhme (1992), *S. gottlebei* has a notably attractive coloration combining white, green, and pink with reddish patches. The known distribution area is limited to the Isalo Massif, central-southern Madagascar (Glaw and Vences, 2007). *Scaphiophryne gottlebei* exhibits fossorial habits by burrowing and hiding underground but is also capable of climbing canyon walls (Mercurio et al., 2008). An initial assessment classified the species as Critically Endangered due to its restricted distribution and the number of threats to it, which included habitat alteration and collection for the inter-

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Emergency procedures in the field: a report of wound treatment and fast healing in the giant ditch frog (Leptodactylus fallax)

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Abstract

*A sub-adult, female, giant ditch frog (*Leptodactylus fallax*) (known locally as a mountain chicken) presented with a puncture of the coelomic cavity with partial intestinal evisceration. Improvised field treatment included replacement of the eviscerated intestines and closure of the wound using cotton-tipped applicator swabs. After seven days, the animal's injury appeared to be healed. During the rapid progression of the healing process, the animal showed no adverse effects. This report demonstrates a novel and successful field technique for wound treatment of a serious penetrating injury in an amphibian. It also shows a practical, feasible, and beneficial procedure that improved this animal's welfare and that might be appropriate under field conditions or if standard medical procedures cannot be followed.*

Keywords: *amphibian medicine, animal welfare, field surgery,* Leptodactylus fallax*, minor surgery, wound healing*

Introduction

When working in the field, researchers are often confronted with situations where veterinary medical procedures are needed, either as part of the project in progress or even in unexpected circumstances. Field surgery is used when it is impractical to transport animals to a laboratory or hospital/rehabilitation centre (Philo *et al* 1981) or when transport would cause more harm. Implantation of transmitters for radiotelemetry studies, and/or tags for identification, are among the most common *in loco* minor surgeries in amphibians (eg Sinsch 1988; Corn 1992; Wright 2001). Besides these, reports on amphibian medical procedures in the field are sparse. In addition, these kinds of actions in the field raise ethical issues. For instance, Kirkwood and Sainsbury (1996) suggested that the decision to intervene to treat (as opposed to euthanasing to prevent further suffering) cases of sick or injured free-living wild animals should not be based on welfare grounds alone but should also consider conservation relevance of a species or population and other factors also.

The giant ditch frog (*Leptodactylus fallax*), known locally as the mountain chicken, is one of the world's largest frogs and currently listed as Critically Endangered (Fa *et al* 2010). Adult individuals may reach more than 1 kg and a maximum snout to vent length (SVL) of 210 mm (Lescure 1979; Garcia *et al* 2007). The species once inhabited at least five major islands in the Lesser Antilles, but occurs now only on two islands: Dominica and Montserrat (Lescure 1979; Schwartz & Henderson 1991; Daltry & Gray 1999; Hedges & Heinicke 2007). Over-hunting, together with habitat loss, and introduction of alien predators are major factors affecting both abundance and distribution (Hedges 1993; Kaiser 1994). However, a new emergent threat, the infectious disease caused by the chytrid fungus (*Batrachochytrium dendrobatidis* [*Bd*]), has been responsible for both pushing the Dominica population to extinction (no positive sightings have taken place in the last few years), and more recently, causing sharp die-offs in Montserrat (Garcia *et al* 2007; Young 2008; Fa *et al* 2010; GM Rosa and A Fernández-Loras, personal observation 2007).

In this short communication we report a minor surgery improvised in the field in a giant ditch frog. We also follow and discuss the healing process under natural conditions.

Description

This work was carried out during ongoing research on the *Bd* outbreak that is threatening the giant ditch frog on Montserrat by Durrell Wildlife Conservation Trust, the Zoological Society of London and Parken Zoo, in collaboration with Montserrat's Forestry Department (Stevens & Waldmann 2001). A sub-adult female *L. fallax* was found within the transect along the Fairy Walk *ghaut*, an area in the Centre Hills used for regular monitoring of long-term

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Research Note

First Reports of Leech Parasitism in Malagasy Anurans

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ABSTRACT: Seven isolated cases of haemadipsid leech parasitism in anuran amphibians living in the rainforests of eastern Madagascar are described. *Boophis madagascariensis*, *Boophis roseipalmatus*, *Gephyromantis luteus*, and *Mantidactylus* sp. aff. *grandidieri* were found to be new host records for *Malagabdella* species, and this study represents the first description of amphibian–leech interactions in Madagascar, the second report of leeches as ectoparasites of Malagasy vertebrates, and the fourth reference of haemadipsid as amphibian parasites.

KEY WORDS: Boophinae, Domanibdelline, ectoparasites, Haemadipsidae, Mantellidae, Mantellinae, Madagascar.

Leech–amphibian interactions have been described for a wide variety of species and have been found to be relatively common in North America (e.g., Siddall and Bowerman, 2006; Graham and Borda, 2010; McCallum et al., 2011), Europe (e.g., Merilä and Sterner, 2002; Ayres and Iglesias, 2008; Tiberti and Gentilli, 2010), and Australia (e.g., Burgin and Schell, 2005). Much of the literature regarding the relationships between these 2 taxonomic groups, however, has been restricted to freshwater leeches acting as ectoparasites of anuran and urodel species from temperate regions, and, to date, very little information is available for their terrestrial counterparts, the haemadipsids (Haemadipsidae), in tropical and subtropical ecosystems.

Madagascar, one of the most important areas for global biodiversity conservation, is home to 5 described terrestrial, sanguivorous leeches (Richardson, 1975; Borda, 2006), 1 freshwater leech (Westergren and Siddall, 2004), and one of the world's richest amphibian faunas with an estimated number of more than 400 anuran species (Vieites et al., 2009). Despite a recent increase in knowledge regarding Madagascar's frogs (e.g., Vieites et al., 2009; Gehring et al., 2010), very few studies have focused on life history or interspecific interactions with other animal groups, and information, when existent, is scarce. The situation is even worse for the island's haemadipsid species, for which, excluding 1 recent exception for lemurs (Wright et al., 2009), virtually no information on biology and ecological interactions is currently available in the scientific literature.

Trophic relationships between leeches and amphibians are complex, and reports range from temporary attachment (Ayres and Iglesias, 2008; Beukema and Pous, 2010), to predation of egg clutches (Trauth and Neal, 2004; Romano et al., 2008), young tadpoles (Berven and Boltz, 2001), and even adults (Merilä and Sterner, 2002). Leeches have been reported to constitute important vectors of amphibian pathogens such as *Ichthyophonus* sp. (Raffel et al., 2006) and anuran trypanosomes causing various diseases (Siddall and Desser, 1992; Readel and Golberg, 2010). They may also indirectly affect the amphibian host by increasing predation risk and lowering fecundity and mating success (Merilä and Sterner, 2002). These invertebrates have also been associated with predator-induced phenotypic variation (Berven and Boltz, 2001; Schalk et al., 2002) and even amphibian population declines (Stead and Pope, 2010).

Herein we present the first reports of haemadipsid leech–amphibian interactions in Madagascar, providing the description and documentation of 7 isolated cases of leech parasitism upon the island's anurans. All observations occurred during a series of herpetological surveys on the east coast of Madagascar between 1992 and 2010. Most of the eastern coastal strip consists of a mosaic of lowland rainforests with an annual rainfall of 2,000–3,000 mm (Green and Sussman, 1990; Primack and Corlett, 2005). Taxonomic identification of the leeches was determined

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ORIGINAL PAPER

The amphibians of the relict Betampona low-elevation rainforest, eastern Madagascar: an application of the integrative taxonomy approach to biodiversity assessments

Gonçalo M. Rosa · Franco Andreone · Angelica Crottini · J. Susanne Hauswaldt · Jean Noël · Nirhy H. Rabibisoa · Miora O. Randriambahiniarime · Rui Rebelo · Christopher J. Raxworthy

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Abstract The Strict Nature Reserve of Betampona protects one of the last remaining relicts (about 2,228 ha) of low elevation rainforests in eastern Madagascar. Yet little has been previously published about the amphibian fauna of this rainforest. During 2004 and 2007, Betampona was surveyed over a total period of 102 days. Frogs were searched by opportunistic searching, pitfall trapping and acoustic surveys. The survey work confirmed the occurrence of 76 taxa, of which 36 are currently candidate species and about 30% were first considered as undescribed species. The identification of species included a multidimensional and integrative approach that links morphology, bioacoustics, ecology and

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Fighting for a leaf: agonistic behaviour in Malagasy frogs of the genus *Guibemantis* (Anura Mantellidae)

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Anuran vocalizations have a broad role in communication. In many cases, some calls help to avoid escalation to aggressive interactions that are energetically costly. The calling activity and agonistic behaviour of a *Guibemantis* sp. aff. *bicalcaratus* are here reported based on observations made at the Réserve Naturelle Intégrale de Betampona, East Madagascar. In addition, we describe a novel "aggressive" call type, produced to challenge territorial intruders.

KEY WORDS: bioacoustics, agonistic behaviour, *Guibemantis*, Madagascar.

Calls in anurans have been the subject of many studies and are known to play an important role in activities such as attracting females, advertising the position of an individual to conspecific males, defending calling sites and announcing a more aggressive behaviour (WELLS 1977, 2007; DUELLMAN & TRUEB 1994; RYAN 2001).

During the breeding season males of numerous species defend their territories or display sites, resorting first to the use of species-specific calls. Advertisement and aggressive calls associated with visual communication usually prevent the escalation to agonistic contacts, reducing energy costs and avoiding physical injury of the individuals involved (KLUGE 1981; ROBERTSON 1986; BASTOS & HADDAD 1996; MARTINS et al. 1998; ABRUNHOSA & WOGEL 2004). However, fighting behaviour has been observed in several anurans (WELLS 1977, 2007). ZANK et al. (2008) suggest that physical interactions are encouraged by the absence of size differences or absence of clear differences in the males' vocalizations (ROBERTSON 1986).

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ORIGINAL PAPER

Can leftovers from predators be reliably used to monitor marine turtle hatchling sex-ratios? The implications of prey selection by ghost crabs

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Abstract In marine turtles, the sex of an individual is determined by temperatures experienced during embryonic development. Gonad histological observation is still the only reliable way to determine hatchling sex, hampering the study of reproduction and of the demographic consequences of context-dependent sex-ratios, a subject of interest in a warming planet. We investigated whether hatchling remains from predation by *Ocypode cursor* can be used to estimate sex-ratio trends in a green turtle rookery at Poilão, Guinea-Bissau (10°52′N, 15°43′W). Sex could be readily determined in 77 and 79% of the predated hatchlings in 2008 and 2009, respectively. By comparing hatchlings killed by crabs, hatchlings accidentally dying on the reefs, and live hatchlings, we show that ghost crabs select the smaller prey, but do not select according to hatchling sex, which is explained by the lack of hatchling size dimorphism in this population. The proportion of male hatchlings was 0.45 ± 0.06 and 0.15 ± 0.06 for early and late-season clutches, respectively, these differences most likely being explained by rainfall. Using leftovers from predation by crabs may be a good solution to non-invasively monitor broad trends in sex-ratios of sea turtles.

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Introduction

The determination of hatchling sex by incubation temperature is a well-known phenomenon that occurs in all extant sea turtles, whereby high incubation temperatures result in the development of more females, while low temperatures yield more males (Wibbels 2003). The effect of temperature on sex determination occurs only in a relatively short period during the whole incubation. This thermo-sensitive period takes place during the second third of incubation, after which hatchling sex becomes determined (Merchant-Larios et al. 1997). The effect of temperature may be dramatic, leading many clutches to develop either 100% males or females, whereas in the so called transitional range of temperatures, a mixture of males and females is produced. Included in this range, there is a temperature at which a 1:1 sex-ratio will be produced—the pivotal temperature (Wibbels 2003).

The potential impacts of the current global warming on sea turtle sex-ratios have become a growing conservation concern (Godley et al. 2002; Glen and Mrosovsky 2004; Chaloupka et al. 2008; Godley 2009; Witt et al. 2010).

Insights on the *Triturus marmoratus* predation upon adult newts

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Key words: *Triturus marmoratus*, diet, energy requirements, reproduction.

Resumen: *Triturus marmoratus* es una de las especies de tritón más grandes dentro de su género, estando su dieta constituida por invertebrados y huevos y larvas de anfibios. A pesar de ello, de manera anecdótica se ha registrado también el consumo de ejemplares adultos de otras especies de tritones. En esta nota presentamos un nuevo caso de consumo de un ejemplar adulto de tritón (*Lissotriton boscai*) por *T. marmoratus* y planteamos la hipótesis de que este consumo sea más frecuente de lo que las evidencias publicadas sugieren.

The marbled newt *Triturus marmoratus* (Latreille, 1800) is one of the largest newts in its genus, with females reaching 16 cm total length (Ferrand de Almeida *et al.*, 2001). This species occurs from western France to north western Portugal (Themudo & Arntzen, 2010), and its life-history differs between populations (Jakob *et al.*, 2002). In highland populations of northern Portugal, *T. marmoratus* hibernates in the winter and aestivates in the summer, moving to water between these periods, exclusively for breeding. In lowlands breeding occurs in late winter and early spring, and individuals remain terrestrial during the rest of the year (Caetano, 1988).

T. marmoratus diet changes throughout life stage and as adult, it varies according to phase (terrestrial *versus* aquatic) (Montori, 2010) and between populations (Villero *et al.*, 2006). Overall, individuals are known to feed on invertebrates such as insect larvae, earthworms, copepods, slugs, snails (Caetano, 1988), and amphibian eggs and larvae (Villero *et al.*, 2006; Diego-Rasilla, 2003). Only three reports exist about *T. marmoratus* preying on adult newts in the Iberian Peninsula: one on alpine newt *Ichthyosaura alpestris* (Orizaola & Rodríguez del Valle, 2000), one on palmate newt *Lissotriton helveticus* (Crespo-Diaz & Sanz-Azkue, 2009), and one on Bosca's newt *Lissotriton boscai* (Ayres, 2007). Similar behaviours are reported for other caudate species (Hagström, 1979; Steward, 1969 in Crespo-Diaz & Sanz-Azkue, 2009).

On 24 May 2013, while monitoring the amphibian community for disease screening at Serra da Estrela Natural Park, Portugal (Rosa *et al.*, 2013), several individuals were captured in a forestry tank (Tanque de Folgosinho, 40°29'37.09"N / 7°31'47.61"W; 1079 masl) using dip-nets. During the handling of an adult female *T. marmoratus*, it regurgitated an adult *L. boscai* (Figure 1).The female measured 67 mm SVL, and the preyed *L. boscai*, 24 mm SVL (Figure 1c).

All records of adult newts' consumption by *T. marmoratus* are reported in aquatic environment. These events may be related to: i) food availability in the water body (Griffiths, 1996; Villero *et al.*, 2006; Gosá & Sarasola, 2010); ii) voracious

BRILL

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CRUSTACEANA

PREDATION ON THE TREEFROG *BOOPHIS RUFIOCULIS* (ANURA, MANTELLIDAE) BY THE FRESHWATER CRAB *HYDROTHELPHUSA* SP. (DECAPODA, POTAMONAUTIDAE) IN MADAGASCAR

BY

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Freshwater crabs are found throughout inland waters within the tropical and warm temperate zones of the world. They produce eggs that develop directly into hatchling crabs, in contrast to their marine relatives who produce thousands of eggs that develop into planktonic larvae. In Madagascar, all primary freshwater crabs that live in the freshwater ecosystems belong to the family Potamonautidae Bott, 1970 (Cumberlidge & Sternberg, 2002; Ng et al., 2008). This monophyletic family is endemic to the Afrotropical region (Cumberlidge, 2008) and includes 142 species within 18 genera, of which the Malagasy freshwater crab fauna comprises 15 species in 7 genera. The genus *Hydrothelphusa* comprises 4 species distinguishable by the morphological character of the carapace, sternum, mouthparts and gonopods (Cumberlidge & Sternberg, 2002; Cumberlidge et al., 2007; Cumberlidge & Meyer, 2009).

With few exceptions, freshwater crabs are generally omnivores, eating vegetable matter, live invertebrate prey, and even small vertebrates (Ng, 1988; Dobson, 2004; Cumberlidge et al., 2009). The predation of anurans by freshwater crabs is mainly centred on the amphibian's eggs and tadpoles (Pyke et al., 2013), even though there are some reports of occasional predation on juveniles and adults (McCormick & Polis, 1982; Toledo, 2005; Hirschmann & Hödl, 2006; Gutsche & Elepfandt, 2007; Caldart et al., 2011; Andrade et al., 2012; Pyke et al., 2013).

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Opportunistic but voracious: Madagascan spiny-tailed iguana, *Oplurus cuvieri* (Reptilia: Opluridae) predation upon a small mammal

Gonçalo M. Rosa[1,2,*] and Sylviane Rakotozafy[3]

The Madagascan spiny-tailed iguana, *Oplurus cuvieri* (Gray, 1831), is an endemic lizard widely distributed in the northwest and western regions of Madagascar. Found at low-to-mid elevations in dry deciduous and transitional forests, this diurnal lizard presents largely arboreal habits (Blanc, 1977), and spends much of its time gripping to trunks and branches, where it remains motionless as it scans the surrounding area for prey (Mori and Randriamahazo, 2002).

This iguanid is one of the exceptional saurian species of Madagascar in that its reproduction, ecological and behavioural aspects have been extensively well documented (see Brillet 1982; Randriamahazo and Mori, 1999, 2001, 2004, 2005; Randriamahazo, 2000; Mori and Randriamahazo, 2002). However, detailed data on natural diet has been poorly studied (Randriamahazo and Mori, 2012). *Oplurus cuvieri* has been considered to be a sit-and-wait predator largely depending on insects (mainly ants) (Brillet, 1982; Mori and Randriamahazo, 2002; Randriamahazo and Mori, 2012), but also occasionally exploits plant matter such as flowers, fruits and leaves by active foraging (Brillet, 1982; Bloxam et al., 1996; Mori and Randriamahazo, 2002; Randriamahazo and Mori, 2012). *Oplurus quadrimaculatus*, a closely related species, is known to occasionally consume small lizards (Brillet, 1982; Cadle, 2003), which we suspect may also be true for *O. cuvieri*. However, no records of ingestion or predation on lizard species have been have been published so far. On the other hand, according to Brillet (1982), *O. cuvieri* also "*accepte les jeunes mammifères*" [takes the *small* mammals], although it is unclear whether this was based on observations from nature or captivity.

Here we report on an adult *Oplurus cuvieri* preying upon a small mammal, *Rattus rattus* (Linnaeus, 1758) in Sahamalaza peninsula, Madagascar. The observation confirms the statement by Brillet (1982) for these iguanids in the wild, where *Oplurus* can prey upon vertebrates (particularly small mammals), and additionally records a new predator for the rodent.

The Sahamalaza peninsula is located on the north-western coast of Madagascar (Mahajanga Province, Sofia Region) and is characterized by a multitude of different habitats from coral reefs to mangroves and dry littoral forests (Projet ZICOMA, 1999, Andreone et al., 2001). The peninsula harbours the Parc National Sahamalaza-Nosy Radama where it is possible to find the endemic lemurs *Eulemur flavifrons* and *Lepilemur sahamalazensis* (Mittermeier et al., 2010). The predation event took place on the 24 January 2013, around 17:00 in Ankarafa forest at the AEECL (Association Européenne pour l'Etude et la Conservation des Lémuriens) field station (14°22.09'S, 47°45.857'E, 160 m a.s.l.). It is an open area for tent-shelter, containing a food store, and is located within a secondary forest fragment.

The lizard suddenly made a fast dash from a resting posture and chased a micro mammal. We did not observe the capture, but the small mammal (a young *Rattus rattus*) was taken away by the *Oplurus* from the site of capture (the food store) to be consumed a few meters away. After seizing the prey by the head, the adult *Oplurus cuvieri* (SVL 135 mm) sat on a rock and started to slam the body of the prey vigorously against the rock (Fig. 1.A). Moving on to a different rock, the lizard slammed its prey against the hard surface another two times until dead (Fig. 1.B). After about 3 minutes, the swallowing process started: with a single

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SCIENTIFIC NOTE

WHERE TO SPEND THE WINTER IN SERRA DA ESTRELA? THE FIRST RECORD OF *DIPHYUS QUADRIPUNCTORIUS* (MUELLER, 1776) OVERWINTERING IN A SUBTERRANEAN HABITAT IN PORTUGAL[1]

Andreia Penado,[2,3] Marta Sampaio,[4] Madalena Madeira,[5] Jesus Selfa,[6] and Gonçalo M. Rosa[4,7]

The family Ichneumonidae is remarkably diverse with at least 100,000 species worldwide (Gauld et al., 2002). Of those, nearly 4,300 species constitute the large subfamily Ichneumoninae which comprises over 440 genera (Yu et al., 2005). Despite this diversity, the Portuguese fauna of this subfamily is still poorly known, with only 45 recorded species (Selfa and Anento, 1998a).

Ichneumonid wasps are important parasitoids, with females laying eggs on or in other insects such as the larvae and pupae of Coleoptera, Raphidioptera, Trichoptera, Hymenoptera and Lepidoptera, plus non-insect Chelicerata (Araneae) (Rasnitsyn and Siitan, 1981; Goulet and Huber, 1993).

Underground habitats can enclose a surprising biodiversity with diverse taxa represented and unique species inhabiting them (Romero, 2009). Although Hymenoptera are not common in underground environments (Moldovan, 2004), some species have been documented to occur in caves during the cooler months (Novak et al., 2010). However, no ichneumonid wasps are known to be cave-obligate (troglobiotic) (Decu et al., 1998; Romero, 2009).

One of the most frequent Ichneumonidae species found in European subterranean environments is *Diphyus quadripunctorius* (Müller, 1776), but its presence in these habitats has been reported only occasionally in France, Serbia, Switzerland, Spain, Germany, Romania, Belgium, Czech Republic, Austria and Slovenia (Jeannel, 1926; Remy, 1953; Jequier, 1961; Selfa and Escolà, 1991; Weber, 1995; Decu et al., 1998; Delhez et al., 1999; Kubátová and Devořák, 2005; Christian and Spötl, 2010; Novak et al., 2010). *Diphyus quadripunctorius*

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Another frog on the menu: predation of *Trachops cirrhosus* (Chiroptera: Phyllostomidae) upon *Osteocephalus oophagus* (Anura: Hylidae)

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Abstract
We report a predation event of the fringe-lipped bat (*Trachops cirrhosus*) (Chiroptera: Phyllostomidae) upon the slender-legged tree frog (*Osteocephalus oophagus*) (Anura: Hylidae), in Central Amazonia, Brazil. This observation adds a new prey item to the *T. cirrhosus* diet and shines some light on the scarce ecological knowledge of both predator and prey species.

Keywords: Chiroptera, Anura, Central Amazonia, trophic relationships.

Introduction

The fringe-lipped bat (*Trachops cirrhosus*) is a medium-sized monotypic bat easily identified by its conspicuous cylindrical, wartlike projections on lips and chin and by the finely serrated margins of the noseleaf (Cramer et al., 2001; Williams & Genoways, 2007). The species is common in most Neotropical dry and moist forest communities (Arita, 1993) and ranges from southern Mexico to the Guianas, Bolivia and southern Brazil, with 3 recognized subspecies: *T. c. cirrhosus* (from Costa Rica to most of the South American distribution), *T. c. ehrhardti* (southern parts of Brazil and Bolivia) and *T. c. coffini* (from Mexico to Nicaragua) (Jones & Carter, 1976; Williams & Genoways, 2007).

T. cirrhosus is considered a foliage gleaning omnivore (Humphrey et al., 1983) and is known to opportunistically feed upon whatever prey it manages to seize and subdue (Pine & Anderson, 1979). Its diet is mainly comprised of insects (Bonato et al., 2004), however, it also consumes a large variety of vertebrates such as birds (Rodrigues et al., 2004), lizards (Goodwin & Greenhall, 1961; Pine & Anderson, 1979; Whitaker & Findley, 1980; Bonato et al., 2004) and small mammals (Peracchi et al., 1982; Arias et al., 1999; Bonato & Facure, 2000; Ferrer et al., 2000; Bonato et al., 2004), as well as fruits and seeds (Whitaker & Findley, 1980; Humphrey et al., 1983). Nevertheless, amphibians are the species' most notorious food item (*T. cirrhosus* is also known as the frog-eating bat), and it is able to tell apart venomous from non-venomous species of frogs by their calls (Ryan & Tuttle, 1983).

Several studies have addressed the interaction between *T. cirrhosus* and anurans in Central and South America where the species is known to prey upon *Engystomops pustulosus* (Barclay et al., 1981; Tuttle & Ryan, 1981; Tuttle et al., 1982) and *Smilisca sila* (Nunes, 1988). However, in the rest of the species' distribution, information is limited to the description of "anurans" as prey in Brazil by Bonato et al., (2004) (based upon the analysis of food remains in the gastrointestinal tracts of museum specimens). An additional record of bats preying upon *Osteocephalus taurinus* was reported for the Surumoni River in Venezuela (Amézquita & Hödl, 2004) and may also be related to *T. cirrhosus*, however, the authors where not able to identify the bat species.

The slender-legged tree frogs of the genus *Osteocephalus* are found from the Guianas to southeastern Brazil and northeastern Argentina and currently 25 species have been described (Jungfer, 2011; AmphibiaWeb, 2012). Three of

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Two remarkable prey items for a chicken: *Leptodactylus fallax* Müller, 1926 predation upon the theraphosid spider *Cyrtopholis femoralis* Pocock, 1903 and the colubrid snake *Liophis juliae* (Cope, 1879)

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Published records of amphibians preying on either large spiders or snakes in the wild are rare; this note documents predation by individuals of one amphibian species on both large spiders and snakes. Mountain chicken frogs (*Leptodactylus fallax*), which are amongst the largest frogs in the world, were observed successfully consuming the theraphosid spider *Cyrtopholis femoralis* on two occasions and attempting to consume another one on a further occasion on Montserrat. They were also found to have consumed the colubrid snake *Liophis juliae* on Dominica. This is the first theraphosid and the fourth snake species identified in the diet of *L. fallax*, and this frog is the first confirmed predator of *C. femoralis*.

Keywords: Colubridae; diet; Dominica; Leptodactylidae; mountain chicken; Montserrat; Theraphosidae; trophic relationship

Records of spiders preying on amphibians are fairly common in scientific literature, even in larval stages. Wolf spiders and tarantulas are among the main frog predators within the Araneae (e.g. McCormick and Polis 1982, Menin et al. 2005; Schulze and Jansen 2010). The opposite scenario appears to be much rarer, however, with very few and only vague records in peer-reviewed papers of predation events by anurans upon large spiders (e.g. Toft 1980).

Accounts of snakes preying on amphibians are also common in scientific literature and in some areas snakes may be the most significant amphibian predators (see review in Wells 2007). Conversely, anurans have only rarely been recorded to feed on snakes (e.g. Branch 1976; Schwartz and Henderson 1991; Duellman and Lizana 1994; Buley 2003), with most remarkable cases of predation upon reptiles being reported for the voracious *Lithobates catesbeianus* (Shaw, 1802) (e.g. Adams and Pearl 2007).

The "mountain chicken" (*Leptodactylus fallax* Müller, 1926), family Leptodactylidae, is among the world's largest anurans, with adult females reaching a snout-vent length

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Figure 2. Common lizard showing right foreleg damage.

Figure 3. Common lizard showing damage to nasal area.

reptiles (viviparous lizards and slow-worms *Anguis fragilis*). Between 20 May 2008 and 18 July 2008, 18 common lizards and 47 slow-worms were captured (during 38 site visits), from an area that would become the quarry 'plant area'.

The 'injured' common lizard was captured under a felt refuge on 1 July 2008, exhibiting normal thermoregulatory behaviour, and showing no obvious signs of suffering. It was released at a receptor area soon afterwards. Figures 1-3 show its injuries. Ongoing monitoring each year since then has not rediscovered the same lizard.

Consideration of these 'injuries' throws up several possible explanations. Pathology is one possibility; perhaps an infection that causes necrosis of the extremities, or a parasitic organism. The nasal damage was superficially reminiscent of the effects of toadfly *Lucilia bufonivora* on common toads *Bufo bufo*, but the lesions seemed to be healed. Frost damage is another possibility. The restriction of necrosis to the anterior extremities, with none evident elsewhere on the body, suggests only partial exposure to frost, however. Another explanation may be partial predation by small rodents, or invertebrates such as ants, consuming parts of the lizard while it hibernated. Alternatively, forestry operations could have caused injuries, which the lizard survived but then became infected or necrotic. Traumatic injury such as this would have probably resulted in less subtle injuries though, and outright death.

Healed head lesions covered with black skin were recently reported from male sand lizards *Lacerta agilis* from Wareham, by Sainsbury *et al.* (2011), but the authors attributed them to male-male combat.

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DRYMOLUBER DICHROUS (northern woodland racer) and ***ANOLIS FUSCOAURATUS*** (slender anole): PREDATION.

Drymoluber dichrous (Peters, 1863) is a ground-dwelling colubrid from northern South America found in both primary and secondary forest (Borges-Nojosa & Lima, 2001). It is active during the day, sleeping in low vegetation at night (Duellman, 1978; Martins & Oliveira, 1998). Stomach contents indicate that the diet of *D. dichrous* is mainly composed of Leptodactylid frogs and lizards of both Sphaerodactylidae and Gymnophthalmidae families (Martins & Oliveira, 1998; Borges-Nojosa & Lima, 2001). It also preys on teiid lizards (e.g. *Ameiva ameiva* and *Kentropyx calcarata*), other snakes and reptile eggs (Martins & Oliveira, 1998; Pinto, 2006). Thus, these studies indicate that *D. dichrous* preys predominantly upon ground dwelling species (but see Duellman [1978]).

The slender anole *Anolis fuscoauratus* has a

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Updated distribution map and additional record for the cryptic leaf-nosed snake, *Langaha madagascariensis* Bonnaterre, 1790 (Serpentes: Lamprophiidae) from Madagascar

Gonçalo M. Rosa[1,2], Jean Noël[3] and Franco Andreone[4]

Langaha (Lamprophiidae) is a genus of arboreal and mainly diurnal snakes endemic to Madagascar. Species are remarkably conspicuous (Fig. 1) by having a protruding scaly appendage at the front of the snout (Bonnaterre, 1790; Glaw and Vences, 2007; Vidal et al., 2008). Three species are currently recognized, *Langaha madagascariensis* Bonnaterre, 1790, *L. alluaudi* Mocquard, 1901 and *L. pseudoalluaudi* Domergue, 1988.

Langaha madagascariensis is the best-known species with localities mainly in the north, on the west coast, and in the southeastern part of the island (e.g. D'Cruze et al., 2007; Glaw and Vences, 2007; Bora et al., 2010). It occurs in dry and wet forest at low elevations (Glaw and Vences, 2007) and individuals are highly cryptic and therefore hard to find. This might compromise the availability of data on the species life history; information on population density is still very scarce (Krysko, 2003, 2005; IUCN, 2011). Recently, two additional records were provided by Gehring et al. (2010) from Madagascar's east coast. The individuals were found in Vohibola and Tampolo forests considerably enlarging the species' known distribution (Fig. 2).

On 3 February 2007, we found *L. madagascariensis* (Fig. 1) on the east coast of Madagascar at Réserve Naturelle Intégrale N. 1 de Betampona (17°55'34.8" S, 49°12'30.0" E, 320 m elevation). The specimen was not collected but, with a spear-shaped appendage and no jutting supraocular scales, its identification leaves no doubt. This record was taken in a strictly protected area roughly located between the forests mentioned by Gehring et al. (2010). The reserve encompasses a 2228 ha fragment of a once extensive evergreen lowland rainforest about 40 km northwest of the major port of Toamasina (Andriampianina and Peyrieras, 1972; Razokiny, 1985). The male individual was located during night time, while resting hanged on the leaves of a small tree (Fig. 1). The surrounding habitat consisted of secondary rainforest on the border of the reserve, next to the village of Rendrirendry. Our observation represents the first finding of *L. madagascariensis* in a protected area in the central eastern coast of the country, which can act as a sanctuary for this species presumably in decline due to ongoing human pressure on lowland forest throughout Madagascar (IUCN, 2011).

The nearest known populations are found at the littoral forests of Tampolo and Vohibola at a distance

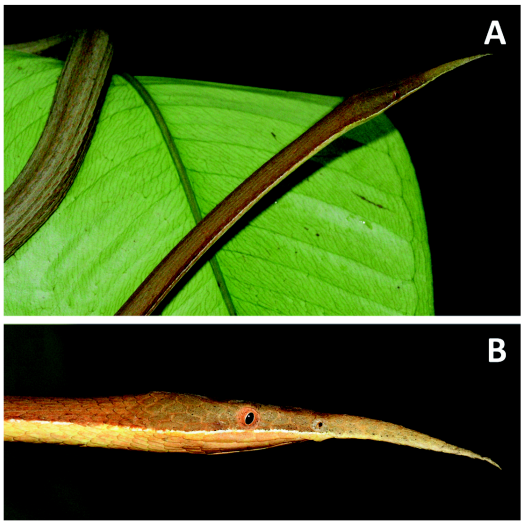

Figure 1. *Langaha madagascariensis* male found at Betampona, eastern Madagascar: (A) individual on a leaf; (B) head close up. Photos: Gonçalo M. Rosa

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Report on the life colouration of the enigmatic burrowing skink *Voeltzkowia rubrocaudata* (Grandidier, 1869) from southwestern Madagascar

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Abstract. *Voeltzkowia* is a monophyletic genus of burrowing skinks endemic to Madagascar. The fossorial habits of these species make them hard to see and study, and witness their life history traits. During two herpetological surveys in southwestern Madagascar (in 2009 and 2011) we found several *Voeltzkowia rubrocaudata* individuals in a corn plantation, a habitat that differs from the forested habitat reported in the literature. Life colouration for this shy scincid is described for the first time.

Key words. Southwestern Madagascar, corn plantation, Squamata, Scincidae.

Voeltzkowia Boettger, 1893 is a poorly known monophyletic genus of scincid lizards endemic of Madagascar (Whiting et al. 2004, Schmitz et al. 2005, Crottini et al. 2009). Species of this genus belong to a phylogenetic clade that contains species of *Amphiglossus* sensu strictu, and the other limb-reduced *Pygomeles* and *Androngo.* With a body elongation and limb reduction, these shy and enigmatic skinks show burrowing habits (Brygoo 1981, Glaw & Vences 2007). The genus is currently composed of three completely limbless species (*V. lineata, V. mira* and *V. rubrocaudata)*, and two species with very rudimentary hindlimbs (*V. petiti* and *V. fierinensis*), but a careful genus revision is currently in act and may increase the number of species of this enigmatic group (A. Miralles, pers. comm.). Despite exhaustive field surveys, fossorial skinks are usually hard to find and some species were not being observed on the island for more than a century (Brygoo 1981, Köhler et al. 2010).

According to the original description, a reddish tail characterizes *Voeltzkowia rubrocaudata.* This species is known from many locations in the arid regions of southwestern and western Madagascar (Grandidier 1869, Glaw & Vences 2007). In this manuscript we report for the first time on the life colouration of live individuals of the species *Voeltzkowia rubrocaudata,* providing also an update on its known distribution and details on habitat preferences.

The individuals reported were found during two herpetological surveys carried out in southwestern Madagascar in late 2009 and early 2011. According to available data summarised by Glaw & Vences (2007), *V. rubrocaudata* is known from Ambohimahalevona, Ampoza, Befandriana, Beroboka Avaratra, Fierin, Isalo, Toliana and Zombitse Forest (currently integrated in the complex Zombitse-Vohibasia National Park). Despite recent herpetological surveys in Zombitse, our team could not confirm its presence. Additionally, intensive research at Isalo National Park (e.g., Mercurio et al. 2008) provided similar results in that area: nevertheless, we observed traces on sand along dry rivers that probably refer to a burrowing skink species.

When pictures of other *Voeltzkowia* species were shown to local people in Andranomaitso (a small village on the Route Nationale 7, Commune rurale de Sakaraha), they informed us that similar animals would be found in the corn plantation near the village (S22° 53' 52.2"– E44° 39' 23.1", 710 m a.s.l.; fig. 1). An intensive search, performed by digging the earthy dry substrate close to the stems and roots, and flipping the rocks of the area, led us to find one individual [MRSN R3726; (corresponding to the tissue sample ACZC2565 where ACZC refers to field numbers of A. Crottini); GenBank accession number JQ005113] on the 11 December 2009 (fig. 2). Such individual (probably a male, SVL 52 mm, with 115 ventral

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SHORT COMMUNICATION

Field observation of foraging behavior by a group of adult diving beetles *Agabus (Gaurodytes) bipustulatus* preying on an adult *Lissotriton boscai*

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Abstract

Adult dytiscids prey on a variety of items including other invertebrates but also larger prey such as frogs and fish. Observations of anuran larvae predation are common. However, there have been no reports concerning predation on caudata post-metamorphosis by dytiscids. Here we describe a predation event by a group of adult diving beetles of the species *Agabus (Gaurodytes) bipustulatus* (Linnaeus, 1767) on an adult *Lissotriton boscai* (Lataste in Tourneville, 1879). This report represents the first observation of foraging behavior of adult diving beetles preying on and consuming a living post-metamorphic newt.

Key words: aquatic invertebrates, Bosca's newt, Dytiscidae, predation, Serra da Estrela, Urodela.

The diving beetle *Agabus bipustulatus* (Linnaeus, 1767) is considered one of the most common and widely distributed in the Palearctic, being found from Europe to the Afro-tropical region, the Near East and North Africa (Nilson & Holmen 1995). It has a great ability to colonize new environments quickly, and occurs in all types of water bodies from small creeks of running water, springs and ponds with little vegetation to shallow lakes with stones or silty bottoms, and cattle troughs (Jackson 1952; Nilson & Holmen 1995). They are often observed in temporary environments where they can cohabit in high densities.

Generally, in early stages, dytiscids feed on *Daphnia* spp. and mosquito larvae, while adults are known for their voracious predation on larger items. Their diet ranges from other insects, gastropods and annelids to vertebrates such as amphibians (particularly tadpoles), and there is also evidence for consumption of fish (Wilson 1923; Drummond & Wolfe 1981). Their polyphagous nature constitutes a survival advantage for living in intermittent streams, characterized by unpredictable changes in environmental conditions, and when particular food resources are unavailable (Fisher *et al.* 1982; Grimm & Fisher 1989).

Many aquatic insect predators are opportunistic feeders, while others select certain sizes or types of prey (Peckarsky 1984). Formanowicz (1986) studied anuran tadpole – aquatic insect predator–prey interactions and found that tadpole vulnerability decreased with increasing tadpole size but was increased by an increasing size of larvae of predaceous diving beetles. However, regardless of their size, most predators appeared to prefer smaller tadpoles (Brodie & Formanowicz 1983).

Reports of predation on urodeles are rare compared to the number of observations on anuran larvae. This is particularly true regarding predation by invertebrates upon post-metamorphic caudata.

The major predators of Bosca's newt *Lissotriton boscai* (Lataste in Tourneville 1879) in the early life stages are invertebrates such as Odonata larvae and adult water beetles (Caetano 1988); however, a predator–prey relationship in post-metamorphic newts has never before been reported. Notwithstanding, cases of invertebrate predation on adult individuals are described for anuran species and even reptiles (Drummond & Wolfe 1981).

In this paper we describe a predation event by a group of diving beetles of the species *Agabus (Gaurodytes) bipustulatus* on an adult *Lissotriton boscai* taking place

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***LEPTODEIRA BAKERI* (Aruban Cat-eyed Snake). PREDATION.** Although predator-prey interactions typically involve vertebrate predators and invertebrate prey, there are several instances of invertebrates preying upon vertebrates (McCormick and Polis 1982. Biol. Rev. 57:29–58). However, many of these events are cases of specific ontogenetic vulnerability of the vertebrate prey, such as the case of aquatic insects preying upon larval amphibians (e.g., Formanowicz 1986. Herpetologica. 42:367–373). Herein, we report a large species of predatory invertebrate preying upon an adult snake.

On 1 July 2010, we observed a large *Scolopendra gigantea* (Amazonian Giant Centipede) consuming an adult *Leptodeira bakeri* within Arikok National Park, Aruba (Fig. 1). When encountered, the centipede had already consumed the flesh surrounding the head and approximately 5 cm of the flesh on the neck of the snake. The centipede was positioned on the trunk of an *Acacia tortuosa* (Twisted Acacia) approximately 0.5 m from the ground. Because of the location and positioning of both the snake and centipede, we interpret the event as a case of predation and not scavenging. Although centipedes are known to prey upon vertebrates (McCormick and Polis, *op. cit.*), and specifically snakes (Easterla 1975. Southwest. Nat. 20:411), many invertebrate-vertebrate encounters have involved nearly equally sized predators and prey. The observation by Easterla (1975, *op. cit.*) refers to a *Scolopendra heros* (Giant Desert Centipede) killing and consuming a juvenile *Rhinocheilus lecontei* (Long-nosed Snake) that was approximately twice its length. Although we did not measure the centipede or the snake, the snake appeared to be approximately three times longer than the centipede. Because both of these species are common on Aruba, this predation event may demonstrate a regular and significant ecological relationship that has been previously overlooked.

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FIG. 1. *Leptodeira bakeri* being consumed by *Scolopendra gigantea* in Arikok National Park, Aruba.

***LIOPHOLIDOPHIS SEXLINEATUS*. DIET.** *Liopholidophis sexlineatus* is frequently found in mountainous areas on the eastern coast of Madagascar (Cadle 1996. Bull. Mus. Comp. Zool. 154:369–464; Glaw and Vences 2007. A Field Guide to the Amphibians and Reptiles of Madagascar, 3rd ed. Vences, Cologne, Germany. 496 pp.; Vences et al. 2002. Ital. J. Zool. 69:263–284). It is a diurnal species with semiaquatic habits, and is thought to feed primarily on frogs (Vences et al., *op. cit.*), including *Ptychadena* spp., *Heterixalus* spp., and *Boophis microtympanum* (Glaw et al. 2007. Zool. Scripta 36:291–300). However, little is known regarding its diet and foraging behavior. Here we document an attempt of predation by a *L. sexlineatus* upon a *Trachylepis madagascariensis*, a common diurnal lizard that inhabits deforested habitats in mountain areas of central Madagascar.

At 1400 h on 5 February 2011, while walking in an area locally known as Andranolava, Andringitra Massif, southeastern Madagascar, (22.152500°S, 46.899222°E, datum: WGS84; elev. about 1970 m), we observed an adult male *L. sexlineatus* in the act of preying upon a *T. madagascariensis* on the ground near the trail (Fig. 1). The snake was wrapped tightly around the skink which had already lost the majority of its tail. The lizard attempted to escape by dislodging the snake using its limbs but ceased struggling after few seconds. The snake adjusted its prey in order to start swallowing. The skink was still alive with its broken tail facing the mouth of the snake. The snake tried to bite the broken tail

PHOTO BY G. M. ROSA

FIG. 1. Predation attempt by a *Liopholidophis sexlineatus* on a *Trachylepis madagascariensis*, in Andringitra National Park, Madagascar.

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Xenopeltis unicolor Boie, 1827 predation upon *Sphenomorphus* sp.

Xenopeltis is a genus of non-venomous snakes characterized by its iridescent, highly polished scales which give to the species the common name "sunbeam snake". Currently, two species are recognized (Bergman, 1955; McDiarmid *et al*., 1999; Rooij, 1917). *Xenopeltis unicolor* Boie, 1827 is found in Southeast Asia and some regions of Indonesia occupying a variety of habitats from primary to secondary forests, agricultural and settled areas from elevations up to 1402 m (Bergman, 1955; Das, 2010; McDiarmid *et al*., 1999; Rooij, 1917). Being nocturnal and subfossorial, this species inhabits burrows excavated by small mammals and crevices within limestones, spending large amounts of time underground on the leaf litter (Das, 2010; McDiarmid *et al*., 1999). Pre-maxillary teeth are found in the aglyphous dentition of *X. unicolor* (Dowling, 1959) enabling a varied diet that consists primarily of frogs, lizards (particularly skinks), small mammals (such as rodents), birds and even other snakes (Bergman, 1955; Das, 2010; McDiarmid *et al*., 1999; Rooij, 1917).

Mertens (1943) described a first predation event in a captive specimen. The author recorded a predation upon a frog being eaten within few seconds. This snake used to be fed every week on a frog, lizard or mouse. In general, the prey was firmed right away being killed by suffocation, or sometimes as much as half an hour later. The frog was then swallowed quickly afterward.

Despite being the most frequently traded species in some areas of Southeast Asia (such as U Minh Thuong National Park, Viet Nam) (Stuart, 2004), its ecology and natural history is still largely unknown (Das, 2004a). Approximately 130 species are currently assigned to the speciose genus *Sphenomorphus* of which 16 can be found in Borneo Island (Das, 2004b; Greer & Shea, 2004; Grismer, 2007). Species of this large group are known to inhabit deep in forests, avoiding sun flecks. However, individuals can climb several meters up tree trunks to avoid predators or even some can seek safety in riparian microhabitats (Inger, 1959; Malkmus, 1991; Inger *et al*., 2001). Montane species tend to be more secretive in their habits which leave them poorly understood (Grismer, 2007). Sympatry is common, particularly in some areas of Borneo, where many species present overlapping distribution (Inger *et al*., 2001).

In this manuscript we describe a predation event by an individual of *Xenopeltis unicolor* upon a *Sphenomorphus* species occurring at the Danum Valley. This report represents a more detailed item in this snake's species diet in the wild as well as a new confirmed predator for these Bornean skinks.

During research work carried out in Borneo Island, on 28 October 2009, an adult individual of *X. unicolor* (TL ±70cm), reddish brown dorsum with white cream ventre, was observed preying on *Sphenomorphus* sp. The predation episode was recorded at the Danum Valley Field Center (at 4°58' N, 118°48' E, 200 m a.s.l.) located on the eastern border of the Danum Valley Conservation Area on the east coast of the Malaysian state of Sabah, Borneo Island. Danum Valley Conservation Area is the largest remaining area of undisturbed lowland (<760m) evergreen forest, where dipterocarps consist of up to 80% of the canopy trees (Marsh & Greer, 1992; Newbery *et al*. 1992). The climate at Danum is equatorial with a mean annual temperature of 26.7 °C and mean relative humidity between 78% and 95% (Marsh & Greer, 1992; Walsh, 1990).

When the team approached the spot (at 10h16am, temp. 28°C and humidity 80%), the *Xenopeltis unicolor* individual was hidden under a rotten log on the leaf litter, near a small stream. The skink was then observed a few seconds later getting closer to the crevice where the snake was lying, without noticing its presence. Suddenly, there was an ambush with the snake appearing under the rubble of the trunk and firmly seizing its prey by the head

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Contributo das metodologias moleculares para a vigilância epidemiológica de doenças infeciosas na fauna selvagem

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SUMÁRIO

A circulação de agentes patogénicos na fauna selvagem tem conduzido a implicações graves na saúde pública, na saúde e no bem-estar animal, e na conservação da Natureza como o atestam as pandemias de síndrome respiratória aguda severa (SARS) e de gripe aviária altamente patogénica (HPAI, associada aos subtipos H5N1 e H7N9) registadas na primeira década do século XXI. As particularidades inerentes à vida livre da fauna selvagem dificultam o estudo e a vigilância epidemiológica dos agentes infeciosos e parasitários, e inviabilizam, muitas vezes, o recurso a métodos convencionais de diagnóstico. As técnicas moleculares apresentam inúmeras vantagens, pois permitem gerar informação epidemiológica que, de outra forma, se manteria desconhecida. Neste capítulo, apresentam-se três estudos de caso sobre agentes tão diversos como fungos, vírus e helmintes em diferentes espécies de hospedeiros, que ilustram o potencial das técnicas moleculares de diagnóstico em epidemiovigilância, gestão e conservação de espécies selvagens.

INTRODUÇÃO

Uma só Saúde

O conceito de "Uma só Saúde" consolidou-se no início do século XXI, a partir da tomada de consciência da interdependência entre a saúde humana, a saúde e o bem-estar animal e a saúde ambiental. Visa a melhoria da qualidade de vida e do bem-estar de todas as espécies e do planeta em geral, através de colaborações interdisciplinares entre a medicina humana, a medicina veterinária e as ciências ambientais[1]. Para essa tomada de consciência, foi essencial a constatação de que a maior parte das doenças infeciosas que afetam o Homem tem origem nos animais. De facto, estima-se que mais de 70% das doenças emergentes tenham origem em animais e que, para a maioria, a fauna selvagem constitua o principal reservatório[2].

Entre outros fatores, o aumento da população humana nos países em desenvolvimento, a destruição e ocupação das zonas naturais, bem como o desenvolvimento dos meios de transporte e crescente facilitação da circulação de pessoas e mercadorias, e a proteção e o consequente incremento das populações de espécies selvagens nos países desenvolvidos, ampliam a interface de contactos viáveis e de trocas de agentes patogénicos entre a fauna selvagem, os animais domésticos e o Homem[2]. O papel da fauna selvagem na ocorrência e na persistência de doenças nas espécies pecuárias pode ter impactos económicos muito grandes, como foi recentemente demonstrado com os anatídeos selvagens na pandemia de gripe aviária[3] e com o javali nos focos de peste suína clássica

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LES AMPHIBIENS DES ZONES ARIDES DE L'OUEST ET DU SUD DE MADAGASCAR

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